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MORPHINE AND THE CANINE BRAIN

The influence of morphine on cerebral perfusion and 5-HT_{2A} receptors

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*You know
I don't
I dream*

Elliott Smith

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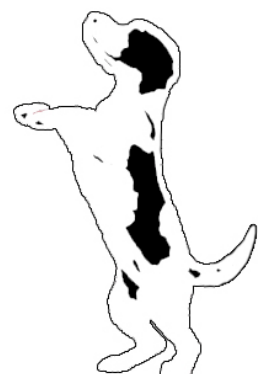
List of Abbreviations

5-HT	5-Hydroxytryptamine, serotonin
ACC	Anterior cingulate cortex
AI	Asymmetry index
BI	Binding index
BW	Body weight
cAMP	Cyclic adenosine monophosphate
CBF	Cerebral blood flow
CO ₂	Carbon dioxide
CSF	Cerebrospinal fluid
DA	Dopamine
DOR	Delta opioid receptor
DRG	Dorsal root ganglion
ECD	Ethylcysteinate dimer
GABA	Gamma-aminobutyric acid
GPCR	G-protein coupled receptor
HMPAO	Hexamethylpropylene amine oxime
HPLC	High pressure liquid chromatography
I	Iodine
IS	Internal standard
IV	Intravenous
KOR	Kappa opioid receptor
LC	Locus coeruleus
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOQ	Limit of quantification
MBq	Megabecquerel
MOR	Mu opioid receptor
NRM	Nucleus raphe magnus
OR	Opioid receptor
PaCO ₂	Partial arterial carbon dioxide pressure
PAG	Periaqueductal gray
PI	Perfusion index
QC	Quality control

List of Abbreviations

rCBF	Regional cerebral blood flow
RVM	Rostroventral medulla
S1	Primary somatosensory cortex
S2	Secondary somatosensory cortex
SD	Standard deviation
SERT	Serotonin transporter
SPECT	Single photon emission computed tomography
SSRI	Selective serotonin reuptake inhibitor
Tc	Technetium
VOI	Volume of interest
WHO	World Health Organization

GENERAL INTRODUCTION



A. Introduction

Providing adequate pain relief (both in humans and animals) remains a challenge even after decades of research and continuous evolutions in pain medication. Major reasons for this are the existing lack in profound understanding of the exact site of action of pain medication in combination with the impaired access to controlled substances, in the case of opioids. The cause for this impaired access to opioids has been studied by the World Health Organization (WHO), which found that there was an imbalance between the prevention of abuse of controlled substances and their use for legitimate medical purposes (WHO, 2011).

According to the WHO, the number of people suffering from inadequately treated or untreated pain is far beyond several millions. These include cancer patients, but also other chronic pain patients and people with severe acute pain (WHO, 2012a and 2012b). In veterinary medicine, chronic pain is also an important issue in cases of cancer and chronic inflammatory processes (Robertson, 2005; Rusbridge and Jeffery, 2008; Fox, 2010; Grubb, 2010a and 2010b).

Better understanding how opioids work and how they interact with the central nervous system can contribute to the expanding research on (equivalent or better) alternative drugs for pain management. Accordingly, in the field of opioids, myriad studies have been conducted (either in human subjects or in animal models) on the topic of problems associated with long-term (ab)use of opioids. As mentioned, animals (rodents mainly, but also higher species) have been used for decades as study models for humans. Additionally, from a veterinary medicine point-of-view, research on pain management in animals as such, has gained interest only in the last decade, leading to a booming research field. Caring for our animals and giving them a good quality of life is becoming more and more important. One of the basic ideas that comes with that is the need to provide efficient pain relief to our animals. This has

been achieved in the past by using drugs that were designed for human use. However, the differences between man and animal and between different animals as well, have given the opportunity to investigate these drugs and their use in animals. Causes for variations in drug action are for instance differences in drug uptake, metabolism, excretion, penetration of natural barriers (e.g. blood brain barrier), etc. Next to pharmacokinetic and –dynamic studies, functional imaging can provide information on specific interactions of drugs with the target organs in vivo and in a non-invasive manner. Single photon emission computed tomography (SPECT) of the brain is one of these functional imaging modalities that is frequently used in drug research.

Over the years, our research group has successfully invested great effort in developing the SPECT technique for its use in small animals and in evaluating several radioligands for brain imaging in dogs. To this day, visualizing the signaling components of the serotonergic (5-HT) and the dopaminergic (DA) system, as well as cerebral blood flow in dogs has led to a rather unique approach to SPECT. On the one hand, progress was made with regards to canine brain pathology, such as behavioral disorders and epilepsy (Peremans et al., 2003b; Martlé et al., 2009). On the other hand, data from these studies have also provided information of great value for human medicine, for example the work done on the serotonin-2A receptors (5-HT_{2A}) (Vermeire et al., 2009).

In addition to the use of SPECT in a neutral, at rest, basal brain state, it is interesting to combine this imaging technique with certain drugs in order to gain insights on how they influence the brain and thus, to better understand how they work. As mentioned previously, next to the evaluation of neurotransmitter systems, regional cerebral blood flow can be estimated. These regional differences in cerebral blood flow are known to be a measure for brain activity (Sokoloff et al., 1977; Gerrits et al., 1998). Therefore, SPECT is a very useful tool to assess the action of different drugs on the brain. In that perspective, this imaging

technique may provide further information on the mode of action of opioids. In this study the effect of morphine on the 5-HT_{2A} receptor and on brain regional perfusion was investigated.

B. Pain modulation and the opioid system

1. Mu opioid receptor agonists: mode of action

Morphine is a potent analgesic derived from the opium poppy, *Papaver somniferum* (Figure 1). It is only after being used (and misused) for a long time that researchers finally unraveled the underlying mode of action of opioids in the 1970s by discovering the opioid receptors. There are three known opioid receptors in man and animal: mu, delta and kappa opioid receptors, i.e. MOR, DOR and KOR respectively (Mansour et al., 1995a; Peckys and Landwehrmeyer, 1998). Based on the type of interaction of the opioid with one or another opioid receptor, opioids can be divided in different groups with different properties: full agonists, partial agonists, agonist/antagonists and full antagonists (Lamont and Matthews, 2007). Morphine belongs to the full MOR agonist group, next to methadone and fentanyl.



Figure 1. The opium poppy *Papaver somniferum* (A) from which the latex is harvested by making incisions in the seed pod (B) and opiate alkaloids like morphine (C) can be extracted.

Opioid receptors are G-protein coupled receptors (GPCRs) that produce inhibitory effects in neurons (review: Connor and Christie, 1999). Receptor activation results in inhibition of adenylyl cyclase causing a decrease in cyclic adenosine monophosphate (cAMP), neuronal hyperpolarization via activation of K⁺ channels, and inhibition of neurotransmitter release via inhibition of Ca⁺⁺ channels (review: Reisine, 1995; Standifer and Pasternak, 1997). The receptors consist of an extracellular ligand-binding part (N-terminus), a transmembranar part

and an intracellular part that interacts with a heterotrimeric G-protein that consists of three subunits, α , β and γ (Figure 2) (review: Forse, 2000).

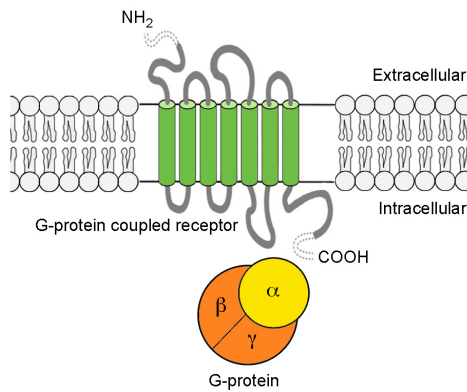


Figure 2. Schematic representation of a G-protein coupled receptor, in this case a mu opioid receptor (MOR).

Opioid receptors are located both on pre- and postsynaptic neuronal membranes as shown by neuronal targeting (Arvidsson et al., 1995; Abbadie et al., 2002). Presynaptically, opioids inhibit the release of neurotransmitters, such as glutamate, gamma-aminobutyric acid (GABA) and substance P, through inhibition of Ca^{++} channels (Bradford et al, 1986; Tanaka and North, 1994; Aicher et al., 2000a and 2000b). Postsynaptically, receptor activation causes opening of G-coupled inwardly rectifying K^+ channels and thus induces hyperpolarization of the cell, preventing excitation and reducing the conductance of action potentials (Grudt and Williams, 1993; Tanaka and North, 1994).

Based on the localization of the ORs, it is clear that opioids have an important role in the transmission, modulation and perception of pain. Opioid receptors can be found in all key sites for nociception, i.e. supraspinally, spinally and in the peripheral nervous system.

The first level at which opioids can modulate nociception, is at the periphery where ORs can be found in the primary afferents and the dorsal root ganglia (DRG) (Atweh and Kuhar, 1977; Arvidsson et al., 1995; Cheng et al., 1996) (Figure 3). At the second, the spinal level ORs are present in the dorsal horn of the spinal cord (Figure 3). Each receptor type has a unique distribution. MORs have a more diffuse distribution in both lamina I and II (Arvidsson et al., 1995), but mainly in lamina II (Ji et al., 1995). The third level at which

opioids modulate nociception, is the brain. The ORs are widely spread throughout the brain and are expressed primarily in the cortex, limbic system and brain stem (Figure 4). Thus, high concentrations of ORs are found in the periaqueductal gray (PAG), the locus coeruleus (LC) and the rostroventral medulla (RVM) (the nucleus raphe magnus (NRM) and the reticular formation) (Kalyuzhny et al., 1996). In most brain regions, all three OR types can be found, albeit in some regions higher numbers of one or the other OR are present. For instance, the mu opioid receptor is mostly present in the amygdala, thalamus, mesencephalon and some brain stem nuclei (review: Le Merrer et al., 2009).

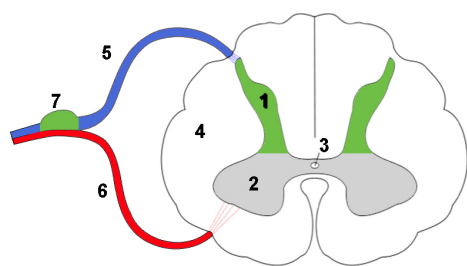


Figure 3. Localization of the mu opioid receptors in the spinal dorsal horn and the dorsal root ganglia (green). 1: dorsal horn; 2: ventral horn; 3: central canal; 4: spinal white matter; 5: dorsal root of spinal nerve; 6: ventral root of spinal nerve; 7: dorsal root ganglion.

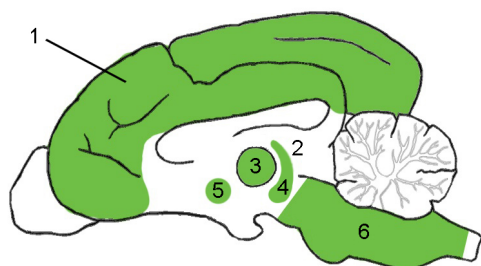


Figure 4. Localization of the mu opioid receptors (MOR) in the brain (green). High densities of the MOR are found in the cerebral cortex (1), the limbic system containing the hippocampus (2), thalamus (3), amygdala (4) and hypothalamus (5), and the brainstem (6).

Since MORs are located in all three of these levels, morphine exerts its analgesic action by modulating neurotransmission at all different levels. In the periphery and in the spinal dorsal horn the activation of MORs reduces the conductance of the painful stimulus (Kline and Wiley, 2008). Supraspinally, MOR activation triggers descending inhibitory pathways (review on descending control of pain: Heinricher et al., 2009). These descending pathways are characterized by the contribution of other neurotransmitter systems, both in the brain itself and in the spinal cord (review: Fields et al., 1991). Those neurotransmitters, such as

serotonin and GABA, are known to mediate in part the analgesic effect of morphine (Nemmani and Mogil, 2003). The contribution of these neurotransmitters in opioid analgesia was investigated by looking for insights on the morphological as well as the electrophysiological and behavioral aspects of these interactions. Since the present work focused on the serotonergic system, more specifically, the 5-HT_{2A} receptor, only the input of the serotonergic system in opioid analgesia will be further discussed.

In rodents, morphological data point to the interaction between the opioid and serotonergic system since there is an anatomical co-distribution of opioid and serotonergic elements. The raphe nuclei contain both encephalinergeric – enkephalin is the endogenous ligand for MORs – and serotonergic neurons (Moss et al., 1981; Bowker and Dilts, 1988). Bulbosapinal 5-HTergic neurons express MOR and, additionally, half of the projection neurons from the RVM to the spinal cord that express MOR are serotonergic (Wang and Wessendorf, 1999).

On top of this, there is a great amount of (electro)physiological and behavioral evidence for the involvement of the serotonergic system in morphine induced antinociception. Two things are important here, first, is serotonergic involvement required for morphine analgesia? And second, which serotonergic receptors are involved? Both of these issues evoked quite some controversy and up to now no clear answer has been found to these questions. The involvement of 5-HT in opioid analgesia has been extensively studied. For instance, raised 5-HT levels and increased 5-HT synthesis were found both in the spinal cord and the brain after systemic administration of morphine in rats and dogs (Godefroy et al., 1981; Bardon and Ruckebusch, 1984; Grauer et al., 1992; Tao and Auerbach, 1994 and 1995). Additionally, next to systemic administration, intraventricular injection of morphine also increased the 5-HT release in the spinal cord (Jung et al., 1994). The increased amount of 5-HT in the spinal cord most likely originates in the higher brain nuclei since local application of morphine to the spinal cord had no effect on the 5-HT levels or the 5-HT turnover (Bineau-Thurotte et al.,

1984; Vasko et al., 1984; Matos et al., 1992). Decreased levels of 5-HT in the central nervous system were shown to reduce morphine-induced analgesia (Vogt, 1974; Proudfit and Anderson, 1975; Bodnar et al., 1981; Berge et al., 1983; Millan and Colpaert, 1991a and 1991b; Zhao et al., 2007).

Several studies were conducted using microinjection techniques where morphine could be applied directly in specific brain regions. Direct administration of morphine in the NRM produces analgesia that is reduced by pretreatment with a 5-HT₂ receptor blocker (Dickenson et al., 1979). Furthermore, administration of morphine directly into the PAG results in antinociception, mediated in part by increased spinal 5-HT levels and the activation of spinal 5-HT_{1A} receptors (Lin et al., 1996; Liu et al., 2002). This latter example leads to the involvement of specific 5-HT receptors in opioid analgesia, where it is not surprising to find different – and sometimes contradictory – results due to the complexity of the excitatory and inhibitory responses to 5-HT by the different 5-HT receptors (Millan, 1995). Reviewing these results would be too extensive, however, it does point towards a possibly important role of these receptors in opioid analgesia and/or opioid side effects.

2. Opioids and their (side)effects

Analgesia is the key property of opioid drugs and is the reason for the great success of these drugs in pain management in both humans and animals. Especially in acute severe cases of pain, opioids are protagonists at achieving profound pain relief. Being the prototypical opioid, morphine is still one of the most administered opioids and its pharmacokinetics in dogs have also been extensively studied. Clinical effective morphine plasma concentrations in dogs are $13,92 \pm 2,39$ ng/mL (KuKanich et al., 2005). In that study it was also reported that a significant analgesic level was present for 2.8 ± 0.6 hours (KuKanich et al., 2005). In a

clinical setting on the other hand, morphine is usually administered every 4 hours to maintain proper analgesia (Lamont and Matthews, 2007).

Due to the widespread distribution of opioid receptors and their endogenous ligands, it is not surprising that, next to the analgesic action of opioids, several adverse effects can occur, both after a short period of administration and after chronic exposure to opioids. These side effects will be briefly discussed in the following section.

Acute side effects

Acute side effects due to opioid administration occurring both in humans and animals include sedation (although sometimes a wanted therapeutic effect in veterinary medicine), nausea, vomiting, respiratory depression (rare but dangerous) and constipation. Most of these effects are dose-related for morphine and other MOR agonists and are clinically manageable. Therefore, the use of opioids has remained one of the cornerstones of acute pain management for decades both in human and veterinary medicine (Lamont and Mathews, 2007; WHO, 2012a and 2012b).

In clinical settings, nausea and vomiting occur frequently in humans and dogs following opioid administration (Lamont and Mathews, 2007; Porreca and Ossipov, 2009). Opioids induce an emetogenic effect via different mechanisms, i.e. the direct stimulation of the chemoreceptor trigger zone, the inhibition of gastrointestinal motility and the stimulation of the vestibular apparatus in the temporal lobe (Porreca and Ossipov, 2009).

Although the effects of opioids on cardiac output, heart rhythm and blood pressure are minimal, high doses of opioids can cause cardiac depression resulting in bradycardia (Maiente et al., 2012; Bowdle, 1998). Due to the diminished responsiveness of respiratory centers in the brain stem, arterial CO₂ concentrations can rise, causing cerebral vasodilation and increased intracranial pressure. Additionally, in the case of morphine, a possible histamine release can provoke vasodilation and hypotension (Barke and Hough, 1993).

Opioid induced respiratory depression can potentially be life threatening and is caused by a combination of several mechanisms including peripheral and central components, i.e. depression of respiratory control centers. Peripherally, opioid receptors are located in the carotid bodies and in the vagal nerves. Additionally, opioid receptors are found on mechanosensory receptors located in the epithelial, submucosal, and muscular layers of the airways that transmit mechanical and sensory information from the lungs (review: Pattinson, 2008).

Problems of constipation due to opioid use are mostly seen in humans (Porreca and Ossipov, 2009). During the perioperative use of opioids in dogs an initial stimulation to defecate followed by ileus, is a typically encountered side effect (Lamont and Mathews, 2007). The inhibition of gastrointestinal motility is caused by central and peripheral opioid receptors, but the effect of opioids on the myenteric plexus – thus inhibiting the intestinal peristaltic movement – is probably the main cause (Porreca and Ossipov, 2009).

Chronic side effects

Long-term use of opioids for chronic pain management is severely hindered by the development of three important side effects: tolerance, addiction and dependence. In research settings, animal models of addiction and dependence have been developed, but the clinical importance of these two side effects is of lesser importance in veterinary medicine compared to their importance in human medicine. The possibility of these side effects is however an important reason for physicians (both veterinary and human) to be cautious with the use of these otherwise very potent analgesics. Nevertheless, in cases of severe pain (e.g. cancer pain, both in humans and animals), the alternative analgesics such as non-steroidal anti-inflammatory drugs are not potent enough to provide sufficient relief for these patients (WHO, 2012a and 2012b). The underlying mechanisms of development of these side effects

are not yet fully understood and better understanding these mechanisms would help in the search for new opioids with fewer side effects or in the search for therapeutic adjuvants that could help to diminish or avoid these side effects.

Tolerance to a drug is defined as a diminished (analgesic) response to the drug or the need for higher doses to achieve the same effect. Morphine induces tolerance to its analgesic effect rather rapidly. There are several hypotheses about how tolerance develops, a diminished capability of inducing receptor internalization and a defective receptor desensitization and endocytosis are most likely the cause (review: Bailey and Connor, 2005; Berger and Whistler, 2010). Normally, after activation of the receptor by a ligand, receptors are rapidly phosphorylated by GPCR kinases (GRKs) and subsequently bound by arrestin. These events uncouple the MOR from G-protein, resulting in receptor desensitization. Endocytosis of the agonist-receptor complex follows the binding of arrestins. In the early endosome, the agonist unbinds, receptors are dephosphorylated and then quickly recycled to the cell surface, resulting in resensitization. The endogenous MOR agonist enkephalin leads to effective MOR internalization and thus receptor resensitization follows in a rapid manner and by analogy, the recovery of response to agonists as well (Figure 5A). With morphine on the other hand, this internalization process is compromised. In a state of tolerance, these mechanisms are defective, causing a deficient response to the ligand (Figure 5B) (review: Berger and Whistler, 2010; Williams et al., 2013).

Other ligands and receptors have been investigated for their possible role in the modulation of opioid receptor internalization. Among these, co-activation of the 5-HT_{2A} receptor has been shown to increase mu opioid receptor internalization (López-Giménez et al., 2008).

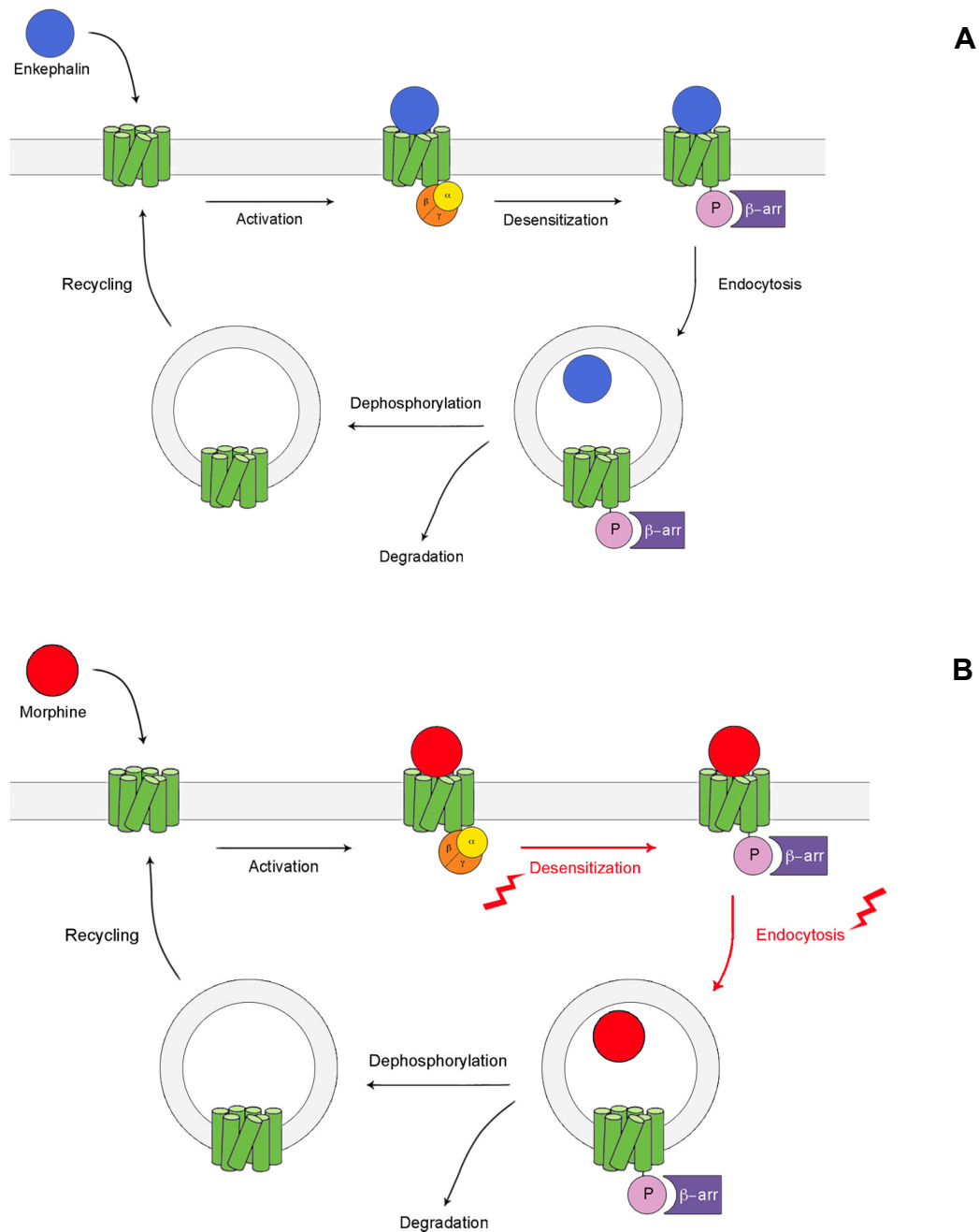


Figure 5. The G-protein coupled receptor (GPCR) cycle. **5A:** After binding of the endogenous agonist enkephalin, the conformation of the mu opioid receptor (MOR) is changed to its activated conformation that binds with the G-protein. Following activation, the MOR is phosphorylated by GPCR kinases that desensitize the receptor and bound by β -arrestin that initiates MOR endocytosis. The MOR can then either be dissociated from β -arrestin, dephosphorylated and recycled to the cell surface; or undergo complete degradation. **5B:** When morphine binds to the MOR, the receptor internalization, desensitization and endocytosis processes are decreased and the normal GPCR cycle is compromised, leading to the complex phenomenon of tolerance.

An addictive state is defined by intense drug craving and compulsive use of the drug. Drug addiction is a very complex phenomenon. It is thought that drugs of abuse abnormally recruit neuronal pathways and neurotransmitter systems responding to natural reinforcement and progressively alter their function. These neurotransmitter systems include the mesolimbic dopaminergic system that promotes reward, pleasure and compulsion and the opioid system that mediates pleasure (review: Koob and Nestler, 1997; Le Merrer et al., 2009). Additionally, drugs of abuse can induce long-lasting neuronal adaptations and a dysfunction of the serotonergic neurons has been observed after repeated administration of drugs of abuse (Salomon et al., 2006). The increased reactivity of these serotonergic neurons was prevented by 5-HT_{2A} receptor antagonists, suggesting an important role for this receptor in the development of addiction (Salomon et al., 2006; Lanteri et al., 2008).

Dependence is defined as an altered physiological state induced by chronic drug exposure, which leads to a withdrawal syndrome upon cessation of drug administration. Chronic drug exposure leads to cellular and molecular adaptations, among which the upregulation of the cAMP pathway. This is an attempt to overcome the chronic inhibition caused by the chronic opioid tone. Once the drug exposure is stopped, the inhibition stops as well, leading to an overdrive of the cAMP system. All of this leads to an overproduction of neurotransmitters, such as noradrenalin, which then causes withdrawal symptoms (Bailey and Connor, 2005). Early symptoms of withdrawal (12-30 hours after cessation of drug intake) include agitation, anxiety, tachycardia, hypertension, muscle aches / tremor, increased tearing, insomnia, runny nose, sweating and yawning. Late symptoms of withdrawal are abdominal cramping, diarrhea, dilated pupils, goose bumps, nausea and vomiting.

C. Pain modulation and brain function

Over the last decades, functional brain imaging has provided useful information on the very complex mechanisms of pain transmission, perception and modulation. Two aspects of brain function can be studied with functional imaging: brain activity by means of cerebral blood flow measurement, and neurochemistry (neurotransmitter systems). Especially the visualization of brain regions that respond to pain with changes in brain activity, and thus blood flow, played a major role in advancing our knowledge of pain (Peyron et al., 2000). However, it is important to note that the activation of a brain region can be the result of the activation of an excitatory neuron or an inhibitory neuron (Sokoloff et al., 1977; Peyron et al., 2000). This cannot be discriminated by brain activity imaging studies alone. The combination of brain activity studies with neurotransmitter studies is therefore of great importance.

1. Brain function and pain

Pain-related functional brain imaging studies typically assess the brain's response to non-painful stimuli compared to painful stimuli. In normal subjects this has led to a better understanding of the brain networks that are involved in the normal processing of pain. The ascending pain pathways and the supraspinal circuitry of pain transmission are to this day the focus of ongoing investigation and the complexity of this matter is beyond the scope of this work (for comprehensive reviews on this topic: Willis and Westlund, 1997; Treede et al., 1999; Ohara et al., 2005).

In healthy subjects undergoing an acute (experimental) painful stimulus the following brain regions were found to be activated in functional brain imaging studies, forming the so-called pain matrix (Legrain et al., 2011): insular cortex, somatosensory cortices (S1 and S2), anterior cingulate cortex (ACC), prefrontal and parietal cortex, thalamus and brainstem

structures (PAG, reticular formation) (Peyron et al., 2000; Casey et al., 1996; Casey et al., 2000). These areas have been shown to process different features of pain (review: Treede et al., 1999; Peyron et al., 2000; Ohara et al., 2005). In short, the sensory-discriminative aspects of pain are processed by the thalamus, both somatosensory areas and the insular cortex. The thalamus is additionally thought to play a role in the arousal reaction to pain. The role of the ACC in pain processing is rather complex as it is involved in the emotional and cognitive-attentional aspects of pain (Peyron et al., 2000). The attentional component of pain perception is also processed in the prefrontal and parietal cortices. The activation of brainstem structures by pain either reflects an arousal reaction to pain or the activation of descending pain inhibitory pathways (Peyron et al., 2000).

The difficulty of interpreting results from studies in pain patients, lies in the great diversity in pain itself. Not only the duration of pain is a factor, the severity and the cause of pain can also vary from case to case. Next to the considerable variation from subject to subject, due to the complexity of pain perception and modulation, even intra-individual variations can occur. All these factors make it difficult to create homogeneous subject groups.

Chronic pain induces neuroplastic changes within the nociceptive system, the so-called peripheral and central sensitization that occur after a chronic noxious stimulus. Tissue damage and inflammation induce sensitization of the peripheral nerve endings thus altering/increasing the responsiveness of the peripheral nociceptors, this is peripheral sensitization (Raja et al., 1988; Muir and Woolf, 2001). This increased nociceptive signaling can induce changes in the synaptic transmission in the spinal cord, this is central sensitization (Woolf, 1996). Both of these mechanisms can cause augmented nociceptive input to the brain, which can cause functional and structural alternations of the pain processing pathways in the brain. This in turn is reflected by altered brain activity patterns in brain imaging studies. Discussing this complex matter (neuroplasticity and additional differences depending

on the type of chronic pain) is beyond the scope of the present work. However, in brief, abnormal over-activation of the lateral pain system (thalamus and parietal cortex) is commonly seen, whereas the responses in the ACC, insula, S1 and prefrontal regions appear to be more complex (Peyron et al., 2000; Seifert and Maihöfner, 2011).

2. Brain function and opioids

The distribution of opioid receptors in the brain clearly indicates that opioids can modulate brain function. As mentioned in the previous part, one of the ways to investigate brain function is by functional imaging of regional cerebral blood flow. Not surprisingly, the influence of opioids on brain function has been studied extensively, mainly with the focus on chronic pain and drug addiction and dependence due to long term opioid (ab-)use.

A first remark on opioids and cerebral blood flow regards the impact of these drugs on global cerebral blood flow. Since opioids can alter respiration, a hypercapnic-induced increase in global cerebral blood flow can occur (Pattinson et al., 2007; MacIntosh et al., 2008). This needs to be taken into account when interpreting imaging results and, if possible, controlling the respiratory rate (and PaCO₂ values) is advised in subjects under anesthesia. This global alteration of blood flow has, however, no correlation to functional brain activity, which is reflected by regional changes in blood flow.

In functional brain imaging studies of healthy subjects receiving only a mu agonist opioid, increases in regional cerebral blood flow were observed in the ACC, the brainstem (including the PAG), the prefrontal and parietal cortex, and the cerebellum, as well as decreased brain activity in the thalamus and posterior cingulate cortex (Firestone et al., 1996; Adler et al., 1997; Casey et al., 2000; Wagner et al., 2001; Khalili-Mahani et al., 2012). In combination with a painful stimulus, opioids tend to decrease the pain-evoked cerebral blood flow augmentation in both cortical and subcortical regions involved in pain processing and/or

modulation. This is in spite of the fact that when given alone, opioids activate certain of these regions (Casey et al., 2000; Wagner et al., 2007). This has been reported in several rodent models of experimental pain as well (review: Thompson and Bushnell, 2012). Additionally, Wagner and colleagues found that an increasing dosage of remifentanyl would increase brain activity in regions involved in the descending inhibition of pain, whereas it would decrease brain activity in regions involved in pain perception (Wagner et al., 2007). However, during combined administration of fentanyl and a painful stimulus, an increased pain-related brain activation of the supplementary motor area and the prefrontal cortex was observed, possibly explained by the blockade of motor function and the interference in the affective-motivational aspect of pain processing by fentanyl (Adler et al., 1997).

D. Pain modulation and the serotonergic system

As mentioned in the previous part, the modulation of pain by opioids relies in part on different neurotransmitter systems and interactions between them. One of these neurotransmitter systems is the serotonergic system. The monoamine serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter widely distributed in the nervous system, both in the periphery and centrally. It is thought to play a role in many varying functions throughout the body, such as mood, platelet aggregation, vascular tone and intestinal motility (review: Mohammad-Zadeh et al., 2008). In human medicine there has been a lot of interest in the involvement of serotonin in various neuropsychiatric diseases and their treatment (e.g. Alzheimer disease, Parkinson disease, anxiety disorders, migraine, depression...) (Graeff et al., 1996; Nikolaus et al., 2009a and 2009b). A role in pain modulation is another important action of 5-HT as a neurotransmitter in the central nervous system. Unlike opioids, the role of 5-HT in pain modulation is not straightforward. Depending on the receptor type and the site of action (as well as the interaction with other neurotransmitter systems) 5-HT was found to have inhibitory or excitatory effects, resp. leading to the attenuation or the enhancement of nociception/pain transmission and sensation (Millan, 1995; Aghajanian and Marek, 1997; Marek and Aghajanian, 1998a; Aghajanian and Marek, 1999; Millan, 2002; Suzuki et al., 2004; Yoshimura and Furue, 2006). However, it can be stated that the overall effect of 5-HT is inhibitory regarding neurotransmission (Grudt et al., 1995).

1. 5-HT receptor subtypes

There are 7 known 5-HT receptor types or families, i.e. from the 5-HT₁ to the 5-HT₇ receptor. Some of these receptors can be subdivided into different subtypes, e.g. 5-HT_{1A}, 1B, 1C and 5-HT_{2A} and 2C (Hoyer et al., 1994). Not all of these receptor (sub)types are

important for nociception, in fact only the receptors belonging to the 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄ and 5-HT₇ receptor families are thought to play a role in pain modulation (Millan, 2002). All 5-HT receptors, except the 5-HT₃ receptor, are GPCRs, although they differ in the effector system activated. 5-HT₁ receptors (with the exception of 5-HT_{1C}) are negatively coupled to adenylate cyclase, whereas 5-HT₄ and 5-HT_{1C} receptors are coupled to adenylate cyclase in an excitatory fashion. 5-HT₂ receptors on the other hand are coupled to the phosphatidylinositol pathway, activating phospholipase C, but also activating phospholipase A₂ (Brodde, 1990; Felder et al., 1990; Hoyer et al., 1994; Berg et al., 1994; Millan, 2002). 5-HT_{2A} receptors are located both pre- and postsynaptically (Doly et al., 2004). The 5-HT₃ receptor is a ligand-gated ion channel and binding to it results in neuronal depolarization and an increase in free intracellular Ca⁺⁺ (review: Reeves and Lummis, 2002).

The source of endogenous 5-HT are the serotonergic neurons that are widely distributed throughout the brain. The great majority of serotonergic cells is located in the different raphe nuclei and the reticular formation (Holmes et al., 1994; Leger et al., 2001; Hornung, 2003). The dorsal and the median raphe nucleus contain most of the 5-HTergic neurons, followed by the nucleus raphe major, obscurus and pallidus (Takeuchi and Sano, 1982; Kojima et al., 1983; Kapadia et al., 1985; Duann et al., 1989; Bjarkam et al., 1997; Gao and Mason, 2001). Two major projections emerge from these nuclei: to the forebrain and to the brainstem and spinal cord (Kazakov et al., 1993; Hornung, 2003). For pain modulation, projections to the spinal cord are particularly important, since 5-HT exerts an antinociceptive action at a spinal level (Furst, 1999; Braz and Basbaum, 2008).

2. 5-HT_{2A} receptor

As mentioned previously, the action of 5-HT is antinociceptive or pronociceptive depending on the receptor type, administration route and pain modality tested (Millan, 2002). The involvement of the 5-HT_{2A} receptor in nociception has been demonstrated in

neuropathic and sustained inflammatory pain states, where administration of 5-HT_{2A} antagonists reduced hyperalgesia (Nitanda et al., 2005; Van Steenwinckel et al., 2008; Thibault et al., 2008). The 5-HT_{2A} receptor is also thought to be involved in the cognitive modulation and the cognitive-evaluative aspects of pain processing due to its location in regions involved in these aspects of pain modulation, i.e. the orbitofrontal, frontal and prefrontal cortex (Kupers et al., 2009).

Next to the role of 5-HT_{2A} receptors in nociception, the focus of research on this receptor was put on its role in neuropsychiatric disorders. Indeed, it has been shown on numerous occasions that the 5-HT_{2A} receptor is involved in either the etiology or the treatment of several mood disorders, such as depression, aggression and anxiety (Celada et al., 2003; Peremans et al., 2003b; Peremans et al., 2005; Nic Dhonnchadha et al., 2003; Meyer et al., 2008; Vermeire et al., 2009). Moreover, it also has been shown to be involved in other neurological diseases such as Parkinson disease, schizophrenia and insomnia (Abbas and Roth, 2008; Li et al., 2010). This has mainly been of interest in human medicine since these diseases are still not fully understood and adequate treatment is often difficult to find. In the past decade however, research involving mood disorders in small animals has progressed as well. Problems of aggression and anxiety in dogs are for instance a growing point of interest in veterinary research. Research on these specific problems has been conducted at our facility over the past years and a link between these mood disorders and the 5-HT_{2A} receptors has been found, similar to that in humans (Vermeire et al., 2009). This is important for two reasons: first, it is important for those dogs and their environment to better understand and treat behaviour problems in dogs; second, the dog can be used as a suitable research model for these diseases since aggression and anxiety occur naturally in dogs, thus providing a better model compared to induced models in other laboratory animals.

E. Methods for the present work: SPECT

Functional imaging of the central nervous system is of great importance, not only to better understand the very complex brain physiology, but also to investigate the underlying causes of various brain pathologies and to gain an insight in the mode of action of centrally acting drugs like opioids. In functional brain imaging, there are two types of studies, namely, studies regarding specific neurotransmitter systems: neuroreceptor binding studies, and the visualization of brain activity, which was discussed earlier. Both are equally important in order to get a full picture of how drugs work and how diseases develop and can be interesting tools to assess ongoing therapy.

Single photon emission computed tomography (SPECT) is one of the conventional functional nuclear imaging techniques. In human medicine, it has proved most useful in the characterization of various diseases, such as Alzheimer's disease, Parkinson disease, epilepsy, psychiatric disease, brain tumors and degenerative diseases. Brain SPECT imaging in small animals has mainly been used in research settings where, beside its value in studying animal models, it has contributed to understanding and underpinning the biological base for behavior disorders from a veterinary medicine point of view (Peremans et al., 2003b; Vermeire et al., 2009). Next to the use of brain SPECT imaging for disease research, brain SPECT can also be used to investigate the mechanism of action of drugs acting on the central nervous system.

1. SPECT Technique

Nuclear functional brain imaging has been an important tool for the investigation of neurological processes. SPECT is an imaging technique used to visualize both neuronal function and components of neurotransmitter systems (receptors, transporters) in vivo in a direct and non-invasive manner using radioactive ligands. This modality is considered to

date, one of the most sensitive modalities to investigate in vivo interactions with neurotransmitters and receptors/transporters (Ametamey et al., 2008).

In short, SPECT is an imaging procedure in which a radioactive isotope (^{123}I , $^{99\text{m}}\text{Tc}$, ^{131}I , ...) bound to a biochemical compound is administered to a subject. As the isotope decays, it emits photons from the body that are detected and recorded by a camera, thus providing an image of the distribution of the radioligand in the body. The major advantage of these radioligands is that the required amount of radiopharmaceutical is subpharmacological, meaning that specific neurotransmitter systems can be visualized without being harmful for the subject. Once the radiopharmaceutical enters the blood stream, it is distributed through the body organs (brain, liver, kidney, heart and the peripheral vascular system) and, according to each organ's affinity for the particular compound, a specific distribution pattern will become visible.

2. Small animal brain imaging

As mentioned, the use of SPECT imaging in small animals has been valuable not only for diseases occurring in these animals, but also for its use as a model for human medicine. Research at our facility has been focusing over the last decades on both aspects. Evidently, perfecting the SPECT technique for its use in small animals was one of the first steps to be accomplished. Using small animals in brain imaging studies comes with some advantages: (1) the possibility to perform longitudinal brain imaging studies (shorter life spans), (2) ex vivo analysis of the brain can possibly be performed after the imaging study, and (3) new drugs can be assessed prior to approval for human use (review: Thompson and Bushnell, 2012).

Unfortunately, there are also some limitations associated with the use of animals. First, and most importantly, is the species difference. This is reflected in several ways: differences

in anatomy, physiology and pathology. Adapting collimator size, developing new software for image reconstruction and creating a canine/feline brain map database were solutions for the anatomy difference. After this, the difference in physiology had to be investigated. Tracers that are commonly used in humans are not necessarily suitable in small animals. Furthermore, differences in permeability of the blood brain barrier can occur between animal species as well. Therefore, feasibility studies first need to be conducted in order to assess the use of each radioligand in different animal species. Finally, using animals as models for human disease has its challenges since common diseases in humans, do not necessarily occur in animals. Inducing these diseases is one way of solving the problem, however, naturally occurring diseases are more likely to provide accurate results. In this light, canine behavioral problems, such as aggression and anxiety, are grateful to work with since they resemble certain neuropsychiatric diseases in humans.

Another important concern when performing small animal brain imaging is the necessity of immobilization of the animal. This requires the use of anesthetics that can have an influence on the central nervous system and could interfere with the studied neurotransmitter system and/or the brain perfusion. In order to eliminate this confounding factor, comparative studies are necessary. So far, no major objections can be made anesthesia-wise when looking at the results from our research group, as long as the used anesthesia protocols are kept constant between studies to ensure optimal comparison and as long as the use of anesthetics is taken into account when interpreting the results.

3. Tracers

The present work has focused on the effects of an opioid, morphine, on the brain perfusion and on the 5-HT_{2A} receptor system in dogs. To assess the influence of morphine on the regional cerebral blood flow, two ^{99m}Tc-labelled tracers were used: ^{99m}Tc-ECD (Ethylcysteinate dimer) and ^{99m}Tc-HMPAO (Hexamethylpropylene amine oxime). For the

evaluation of the influence of morphine on the 5-HT_{2A} receptor system, ¹²³I-labelled R91150 was used.

Cerebral blood flow: ^{99m}Tc-ECD and ^{99m}Tc-HMPAO

Regional brain perfusion is known to correlate with brain activity, making these tracers markers for regional brain activity next to markers for regional perfusion (Sokoloff et al., 1977; Gerrits et al., 1998). Indeed, higher activity means a higher metabolization rate, requiring higher supplies of oxygen, precursors for protein synthesis and glucose, and therefore blood supply.

Even though the first-pass extraction in brain tissue of both radioligands is high, each radioligand has a different retention mechanism. Both tracers undergo intracellular metabolization from a lipophilic form that readily passes the blood-brain-barrier, to a hydrophilic form that is trapped within the cell. However, this trapping mechanism differs between the two tracers. ^{99m}Tc-ECD is enzymatically transformed by intracellular esterases, whereas transformation of ^{99m}Tc-HMPAO is mainly thought to depend on the glutathione content and the redox equilibrium in the cell (Jacquier-Sarlin et al., 1996a and 1996b). In humans, this difference in retention mechanism has been suggested to be a possible cause for differences in regional distribution between both tracers.

^{99m}Tc-ECD has been used in dogs in several studies (Peremans et al., 2001; Peremans et al., 2003b; Martlé et al., 2009; Waelbers et al., 2011 and 2012), however, due to unforeseen technical reasons, the production of ^{99m}Tc-ECD was stopped, therefore creating the need to explore other regional cerebral blood flow tracers, such as ^{99m}Tc-HMPAO. ^{99m}Tc-HMPAO is used frequently in humans, but information on the use of this tracer in dogs is scarce (Bok et al., 1987; Susskind et al., 1996). Hence, a first study was conducted to assess its suitability

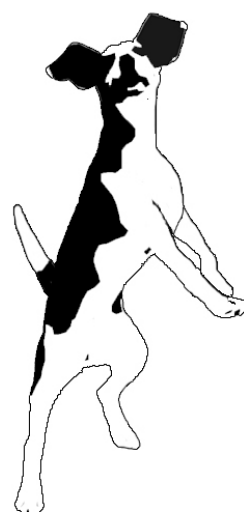
for regional cerebral blood flow evaluation in dogs as well as to compare ^{99m}Tc -ECD and ^{99m}Tc -HMPAO.

5-HT_{2A} receptor system: ^{123}I -R91150

To evaluate the influence of morphine on the 5-HT_{2A} receptor system, the ^{123}I -labelled R91150 tracer was used. This tracer has been used in dogs previously (Peremans et al., 2002a, 2002b and 2003a), including in studies involving canine aggression and anxiety (Peremans et al., 2003b and 2005; Vermeire et al., 2009).

^{123}I -R91150 is synthesized by electrophilic radioiodination on the 5-position of the methoxybenzamide group of R91150, followed by purification with high-performance liquid chromatography. The radioligand is a 5-HT_{2A} antagonist with high affinity ($K_d = 0.11$ nM) and selectivity for the 5-HT_{2A} receptor. The selectivity of the ligand for 5-HT_{2A} receptors with regard to other neurotransmitter receptors such as other 5-HT receptors, including 5-HT_{2C} and 5-HT_{1A}, dopamine receptors, adrenergic receptors and histamine receptors is at least a factor 50. The tracer is displaceable with ketanserin, a 5-HT₂ antagonist and has been previously shown to be suitable for use in dogs (Mertens et al., 1994, Peremans et al., 2002a; Catafau et al., 2006).

SCIENTIFIC AIMS



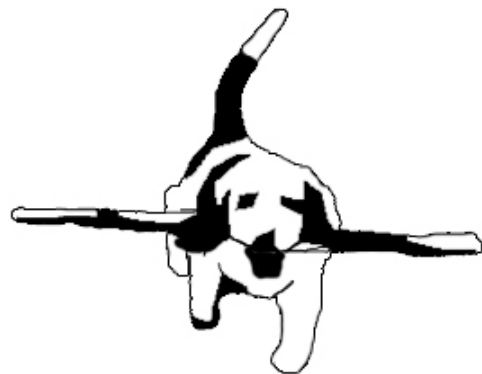
To assess the regional cerebral blood flow the use of functional brain imaging, in this case SPECT, has been very useful. In human medicine two radiolabelled cerebral blood flow tracers are commonly used: ^{99m}Tc -ECD (Ethyl cysteinate dimer) and ^{99m}Tc -HMPAO (Hexamethylpropylene amine oxime). Even though both are equally valuable for regional cerebral blood flow evaluation, differences in distribution patterns between both tracers have been observed. In veterinary medicine ^{99m}Tc -ECD has been the most frequently used cerebral blood flow tracer and comparison between ^{99m}Tc -ECD and ^{99m}Tc -HMPAO is lacking. Therefore the **first aim** of this doctoral thesis was to assess the use of ^{99m}Tc -HMPAO in dogs and the comparison with ^{99m}Tc -ECD.

Evaluation of regional cerebral blood flow is very valuable to gain insights into the mode of action of certain drugs with a supraspinal action. Analgesics like opioids are particularly interesting since they are still the most potent analgesics and are frequently used despite their severe side effects. Better understanding their mode of action could therefore improve their use and possibly help in the search for new and improved analgesics. The **second aim** of this doctoral thesis was therefore to evaluate the effect of a prototypical opioid, morphine, on the regional cerebral blood flow with the functional brain imaging technique SPECT. In a clinical setting analgesia is often required for longer periods and therefore the **third aim** was to assess the influence of a prolonged administration of morphine, consisting of 10 days of oral morphine, on the regional cerebral blood flow.

Opioid analgesics are known to exert their analgesic effect in part through the interaction with other neurotransmitter systems, such as the serotonergic system. The serotonergic system is not only important for the analgesic action of opioids, in this case morphine, it is also believed to play a role in the side effects of opioids. Conversely, opioids have been attributed a potentially antidepressant effect, probably due in part to this interaction with the serotonergic system. One 5-HT receptor has been shown to be involved in all these aspects,

namely the 5-HT_{2A} receptor which has been a central point of interest in mood disorder research. Functional imaging of this receptor by means of SPECT has been previously validated in dogs. The **fourth aim** of this doctoral study was to assess the effect of a single bolus of morphine on the canine cerebral 5-HT_{2A} receptor system. As mentioned in the previous paragraph, analgesia – and certainly mood disorder therapy – require treatment over a prolonged period. Therefore, we also investigated the 5-HT_{2A} receptor binding after a prolonged exposure to morphine, which was the **fifth aim** of this doctoral study.

RESEARCH STUDIES



CHAPTER 1: The comparison of rCBF evaluation with ^{99m}Tc -ECD and ^{99m}Tc -HMPAO in dogs



Adapted from: Adriaens A, Polis I, Waelbers T, Vandermeulen E, Dobbeleir A, De Spiegeleer B, Peremans K. Normal regional distribution of cerebral blood flow in dogs: comparison between (99m) Tc-ethylcysteinate dimer and (99m) Tc-hexamethylpropylene amine oxime single photon emission computed tomography. Vet Radiol Ultrasound. 2013a; 54(4):403-407.

A. Abstract

Two ^{99m}Tc -labelled single photon emission computed tomography (SPECT) cerebral blood flow tracers - ethylcysteinate dimer (ECD) and hexamethylpropylene amine oxime (HMPAO) - are commonly used in human medicine and have been used previously in dogs, but intra-subject comparison of both tracers in dogs is lacking. Therefore, this study investigated whether regional distribution differences between both tracers occur in dogs as is reported in humans.

Methods: Eight beagles underwent two SPECT examinations first with ^{99m}Tc -ECD and followed by ^{99m}Tc -HMPAO. SPECT scanning was performed with a triple head gamma camera equipped with ultrahigh resolution parallel hole collimators. Images were reconstructed using filtered backprojection with a Butterworth filter. Emission data were fitted to a template permitting semi-quantification using predefined regions or volumes of interest (VOI). For each VOI, perfusion indices were calculated by normalizing the regional counts per voxel to total brain counts per voxel. The obtained perfusion indices for each region for both tracers were compared with a paired Student's T-test.

Results: Significant regional differences between both tracers were seen in the subcortical region and to a lesser extent in the cerebellum.

Conclusions: Both tracers can be used to visualize regional cerebral blood flow in dogs, however, due to the observed regional differences, they are not entirely interchangeable.

B. Introduction

Progress in functional brain imaging in dogs is of great importance since these types of brain imaging contribute to detecting functional brain dysfunctions, rather than anatomical abnormalities. This is valuable in the case of canine behavioral problems like anxiety and aggression, and some cases of epilepsy (Peremans et al., 2003b; Martlé et al., 2009; Vermeire et al., 2009). Using diffusible radiolabelled tracers, regional cerebral blood flow (rCBF) can be measured with single photon emission computed tomography (SPECT). Frequently used radioligands include ^{99m}Tc -ECD (ethylcysteinate dimer) and ^{99m}Tc -HMPAO (hexamethylpropylene amine oxime), both commonly used in investigations of neuropsychiatric disorders in human medicine (Kapucu et al., 2009). SPECT is, next to positron emission tomography (PET), a nuclear functional imaging modality that permits the visualization of different neurotransmitter systems in vivo in a non-invasive manner. The major advantages of SPECT over PET are that it is less expensive, more accessible and better suited for the use in animals due to the longer half-lives of the isotopes that permit a longer injection-acquisition interval. In human medicine, the use of SPECT as a diagnostic tool and research modality is widespread (Kapucu et al., 2009). In veterinary medicine, its use is mostly applied in research studies focusing either on animal disease or on the animal as a model for human disease, including research on behavioral problems, epilepsy and the mode of action of several drugs (Peremans et al., 2003b; Martlé et al., 2009; Vermeire et al., 2009; Waelbers et al., 2011).

Even though the first-pass extraction in brain tissue of both radioligands is high, each radioligand has a different retention mechanism. Both tracers undergo intracellular metabolism from a lipophilic form that readily passes the blood-brain-barrier, to a hydrophilic form that is trapped within the cell. However, this trapping mechanism differs

between the two tracers. ^{99m}Tc -ECD is enzymatically transformed by intracellular esterases, whereas transformation of ^{99m}Tc -HMPAO is mainly thought to depend on the glutathione content and the redox equilibrium in the cell (Jacquier-Sarlin et al., 1996a and 1996b). The different retention mechanism has been suggested to be a possible cause for differences in regional distribution between both tracers (Koyama et al., 1997; Oku et al., 1997; Patterson et al., 1997; Siennicki-Lantz et al., 1999).

In human medicine, many investigators have compared the differences between ^{99m}Tc -ECD and ^{99m}Tc -HMPAO in terms of image quality and pharmacokinetics and as a result, differences in rCBF between both tracers were also investigated and revealed regional distribution differences between both tracers in healthy volunteers and in patients with neurological disease (Koyama et al., 1997; Oku et al., 1997; Patterson et al., 1997; Asenbaum et al., 1998; Siennicki-Lantz et al., 1999; Hyun et al., 2001; Ito et al., 2006). In dogs however, both tracers have not yet been compared. Investigating whether the differences seen in humans occur in dogs as well, could contribute to a better rCBF evaluation with either one of these neuroperfusion tracers in dogs. The aim of this study was therefore to compare rCBF evaluation with ^{99m}Tc -ECD and ^{99m}Tc -HMPAO in dogs.

C. Materials and Methods

1. Animals

The study was approved by the Ghent University Ethical Committee (EC 2011-130). All guidelines for animal welfare, imposed by the Ethical Committee, were respected. Eight female neutered purpose bred Beagle dogs (age 5.11 ± 0.34 years, weight 8.86 ± 0.88 kg (mean \pm standard deviation, SD)) were used for this study. None of the dogs had a history of major disease or neurological disorder.

2. Study Design

To evaluate the rCBF, SPECT scans were conducted using ^{99m}Tc -ECD (Neurolite[®], Bristol-Myers Squibb Medical Imaging) and ^{99m}Tc -HMPAO (Ceretec[®], GE Healthcare), both prepared according to the manufacturer's instructions. The first scan was conducted after an intravenous (IV) injection of 747.91 ± 30.22 MBq ^{99m}Tc -ECD. The second scan was performed after an IV injection of 358.95 ± 7.08 MBq ^{99m}Tc -HMPAO. All 8 dogs were scanned with both radioligands in a non-randomized order with a time interval of 6 months.

3. Anesthetic Protocol

Anesthesia is required for SPECT scanning in animals and all scans were performed with the same anesthetic protocol. Premedication (intramuscular dexmedetomidine $375 \mu\text{g}/\text{m}^2$ body surface area, Dexdomitor[®], Orion Corporation, Espoo, Finland) was administered once the plateau was reached for each tracer, i.e. 15 min post tracer injection for ^{99m}Tc -ECD and 10 min post ^{99m}Tc -HMPAO injection (Susskind et al., 1996; Waelbers et al., 2012). Anesthesia was induced by propofol (1.74 ± 0.51 mg/kg intravenously, Propofol[®], Abbott Laboratories, Queenborough, United Kingdom) and maintained with isoflurane (Isoflo[®], Abbott Laboratories) in oxygen. In order to limit the influence of the PaCO_2 on cerebral perfusion, the dogs were mechanically ventilated during anesthesia to preserve eucapnia, i.e. end tidal CO_2 within 35-45 mmHg.

4. Image Acquisition

The dogs were positioned in ventral recumbency with the head stabilized in a preformed cushion, to prevent individual positioning artifacts. Camera and table positioning were then recorded to ensure optimal intra-dog comparisons. The detectors of the triple headed camera were positioned as closely to the dog's head as possible. Image acquisition was started 10

minutes after induction of anesthesia, i.e. 40 min after tracer injection for ^{99m}Tc -ECD and 35 min after tracer injection for ^{99m}Tc -HMPAO. The SPECT scans were performed using a triple head gamma camera (Triad Trionix, Twinsburg, OH, USA) equipped with ultrahigh resolution parallel hole collimators (tomographic resolution, 8 mm full width half maximum). For each acquisition, 120 projection images were obtained on a 128x128 matrix using a step-and-shoot mode (3° steps). Pixel size was 1.72 mm. To make up for the difference in injected activity between both tracers, different scanning times were used, i.e. 10 s per step for ^{99m}Tc -ECD and 15 s per step for ^{99m}Tc -HMPAO. This resulted in a total scanning time of 20 min for ^{99m}Tc -ECD and 30 min for ^{99m}Tc -HMPAO.

5. Image Processing

The acquired images need reconstruction which was performed by using filtered back projection and a Butterworth filter (cut-off 1.6 cycles/cm, order 5).

After this, the emission data were fitted to a template using BRASS software (Brain Registration and Automated SPECT Semiquantification, Nuclear Diagnostics, Stockholm, Sweden) that allows scaling, rotating, and translating of the acquired images in all 3 dimensions. This software was initially developed for humans but the template and regions were created in house for use in dogs (Peremans et al., 2003b). The template provides the necessary anatomical references and consists of a predefined region map with the following volumes-of-interest (VOI): right and left frontal, temporal, parietal and occipital cortices, and subcortical and cerebellar regions. Data from SPECT scans can be semi-quantified with this template by normalizing the counts per pixel in a certain VOI to the counts per pixel of the entire brain (sum of all VOIs). Accordingly, perfusion indices of the different VOIs were calculated as the mean count per voxel in each VOI normalized to the mean total count per total voxel. Normalizing the regional activity to total brain activity has the additional advantage of accounting for the difference in injected radioactivity since the amount of tracer

will be distributed in an equal way over the different regions and the mean counts will increase equally in all regions.

Asymmetry indices (AI) were also compared for the different VOIs, as well as for the right and left hemisphere as a whole. AIs were determined as follows: [(perfusion index of the right VOI or hemisphere – perfusion index of the left VOI or hemisphere) / (perfusion index of the right VOI or hemisphere + perfusion index of the left VOI or hemisphere)] x 200. Finally, a cortical-subcortical index was calculated as (perfusion index of all cortical regions) / (perfusion index of the subcortical region).

6. Statistical analysis

Statistical analysis was performed by the first author using commercial software (SPSS Statistics 19, SPSS Inc, USA). The means \pm SD of the perfusion indices, asymmetry indices and cortical-subcortical ratios were calculated. Normally distributed perfusion index means were compared using the paired Student's T-test. Asymmetry indices and cortical-subcortical ratios were compared using the Wilcoxon signed ranks test. Statistical significance was set at $P < 0.05$. Additionally, correction for multiple comparisons was applied using Bonferroni correction ($P < 0.005$).

D. Results

Perfusion indices (mean \pm SD) and P -values for all brain regions using ^{99m}Tc -ECD and ^{99m}Tc -HMPAO are summarized in Table 1.

Table 1. Perfusion indices for ten brain volumes of interest using ^{99m}Tc -ECD and ^{99m}Tc -HMPAO in eight healthy dogs (mean \pm SD).

Brain region	^{99m}Tc -ECD	^{99m}Tc -HMPAO	P
Left frontal	1.12 \pm 0.05	1.14 \pm 0.04	0.28
Right frontal	1.12 \pm 0.02	1.14 \pm 0.06	0.46
Left temporal	0.86 \pm 0.02	0.88 \pm 0.04	0.16
Right temporal	0.85 \pm 0.05	0.86 \pm 0.04	0.52
Left parietal	1.12 \pm 0.07	1.12 \pm 0.05	0.98
Right parietal	1.11 \pm 0.04	1.09 \pm 0.05	0.29
Left occipital	1.08 \pm 0.07	1.09 \pm 0.07	0.72
Right occipital	1.07 \pm 0.05	1.09 \pm 0.07	0.41
Subcortical	1.24 \pm 0.03	1.17 \pm 0.02	0.001*
Cerebellum	1.05 \pm 0.02	1.09 \pm 0.04	0.03

* $P < 0.005$ after Bonferroni correction for multiple comparisons.

The use of ^{99m}Tc -ECD resulted in higher perfusion indices in the subcortical region compared to ^{99m}Tc -HMPAO ($P < 0.005$). Though no longer significant after correcting for multiple comparisons, perfusion indices in the cerebellum were lower in the ^{99m}Tc -ECD data compared to the ^{99m}Tc -HMPAO data. These differences are illustrated in Figure 1.

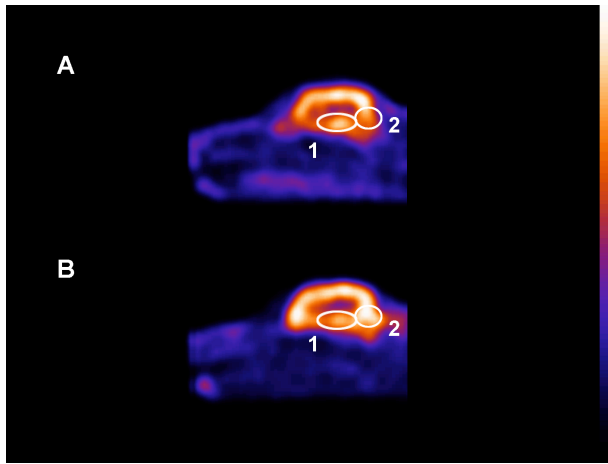


Figure 1. Sagittal acquisitions obtained with ^{99m}Tc -ECD (A) and ^{99m}Tc -HMPAO (B). Higher ^{99m}Tc -ECD uptake was found in the subcortical region (1), whereas the cerebellum (2) showed higher ^{99m}Tc -HMPAO uptake.

Interhemispheric asymmetry was absent for both tracers and AIs for the separate cortical regions did not differ between both tracers (Table 2). The cortical-subcortical ratio was significantly higher for the ^{99m}Tc -HMPAO data (Table 2 and Figure 2).

Table 2. Asymmetry indices for the five cerebral cortical brain regions and the cortical-subcortical ratios obtained with ^{99m}Tc -ECD and ^{99m}Tc -HMPAO in eight healthy dogs (mean \pm SD).

Variable	^{99m}Tc -ECD	^{99m}Tc -HMPAO	<i>P</i>
AI frontal	-0.24 ± 4.47	-0.59 ± 5.54	0.89
AI temporal	-1.30 ± 4.84	-2.80 ± 5.12	0.58
AI parietal	-1.39 ± 6.63	-3.19 ± 4.29	0.33
AI occipital	-0.66 ± 4.32	-0.14 ± 6.34	0.67
AI hemispheres	-0.81 ± 4.20	-1.57 ± 4.88	0.89
CSC	82.15 ± 2.50	87.72 ± 0.77	0.01

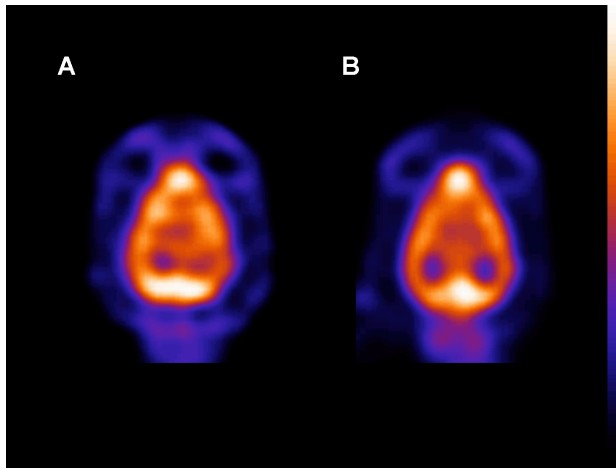


Figure 2. Dorsal acquisitions obtained with ^{99m}Tc -ECD (A) and ^{99m}Tc -HMPAO (B). A higher cortical-subcortical ratio was found for ^{99m}Tc -HMPAO, which can be visualized as better cortical-subcortical contrast.

E. Discussion

This study compared regional ^{99m}Tc -ECD and ^{99m}Tc -HMPAO uptake in the normal canine brain. The data demonstrated a difference in regional uptake between both tracers in the subcortical region and to a lesser extent in the cerebellum.

Reports on rCBF in dogs mainly used ^{99m}Tc -ECD as a tracer (Peremans et al., 2001). Indeed, next to distribution studies in healthy dogs, the use of ^{99m}Tc -ECD has also been documented in research on behavioral problems such as anxiety and aggression and on the influence of several anesthetic protocols on brain perfusion (Peremans et al., 2003b; Vermeire et al., 2009; Waelbers et al., 2011). In contrast, only few studies report on the use of ^{99m}Tc -HMPAO in dogs for rCBF evaluation, mainly focusing on the tracer kinetics (Bok et al., 1987; Susskind et al., 1996). According to those studies, some drawbacks of ^{99m}Tc -HMPAO include a slower blood clearance, resulting in a poorer brain-to-background contrast, a higher extracerebral uptake and higher brain-to-blood back diffusion (Bok et al., 1987; Susskind et al., 1996). Therefore, ^{99m}Tc -HMPAO image quality is considered to be inferior to ^{99m}Tc -ECD images. In the present study, similar to ^{99m}Tc -ECD, the highest perfusion index was found in the subcortical region with ^{99m}Tc -HMPAO as well (Peremans et al., 2001). This is in agreement with the high thalamic ^{99m}Tc -HMPAO uptake found in dogs

(Susskind et al., 1996). High cerebral blood flow was also seen in the frontal cortex for both tracers. This is not in agreement with the study on normal brain perfusion measured with ^{99m}Tc -ECD where frontal perfusion indices were not as high as those seen in the present study (Peremans et al., 2001). An explanation for this discrepancy may be the uniform attenuation correction applied on the data in the latter study, possibly overcorrecting the activity in the less attenuated frontal cortical areas.

In this study we demonstrated that, similar to humans, differences in regional uptake between ^{99m}Tc -ECD and ^{99m}Tc -HMPAO occur in dogs. Remarkably, we found a higher cortical-subcortical ratio for the ^{99m}Tc -HMPAO scans compared to the ^{99m}Tc -ECD scans. This relatively higher accumulation of ^{99m}Tc -HMPAO in the cortical regions seems to be in contrast to the findings in human studies where, globally, ^{99m}Tc -ECD gave better visualization of cortical regions and ^{99m}Tc -HMPAO of subcortical structures (Koyama et al., 1997; Oku et al., 1997; Patterson et al., 1997; Siennicki-Lantz et al., 1999; Ito et al., 2006).

We also found a higher uptake of ^{99m}Tc -HMPAO in the cerebellum compared to ^{99m}Tc -ECD. This is in agreement with several studies in humans (Koyama et al., 1997; Oku et al., 1997; Patterson et al., 1997). As the cerebellum is commonly used as a reference region for semiquantification purposes, this difference in cerebellar uptake is very important and semiquantification using the entire brain, instead of the cerebellum, as a reference region is preferable.

Several hypotheses have been proposed to explain the observed differences in perfusion patterns between ^{99m}Tc -ECD and ^{99m}Tc -HMPAO. Most of these hypotheses depart from different pharmacokinetic properties, which are thought to be the main cause for the observed discrepancies (Oku et al., 1997; Asenbaum et al., 1998; Hyun et al., 2001). First, both tracers have a different trapping mechanism. ^{99m}Tc -ECD trapping depends on esterase activity, whereas ^{99m}Tc -HMPAO trapping depends on glutathione availability and redox equilibrium

(Jacquier-Sarlin et al., 1996a and 1996b). Furthermore, it has been suggested that ^{99m}Tc -ECD was more sensitive to the regional metabolic rate than ^{99m}Tc -HMPAO (Oku et al., 1997). Differences in regional metabolism could therefore influence the accumulation of ^{99m}Tc -ECD more than ^{99m}Tc -HMPAO. Finally, ^{99m}Tc -ECD shows a lower back diffusion from brain tissue to the blood and possesses a higher retained fraction in the brain, compared to ^{99m}Tc -HMPAO that has a significant cerebral blood flow-dependent back diffusion (Andersen et al., 1988; Lassen et al., 1988; Murase et al., 1992; Friberg et al., 1994; Shishido et al., 1994; Yonekura et al., 1994).

Repeatability and reproducibility of ^{99m}Tc -ECD SPECT in dogs have been investigated by our research group in previous studies and were found to be highly satisfactory (Waelbers et al., 2012). For ^{99m}Tc -HMPAO, data in man provided similar results with high reproducibility when data were normalized (Jonsson et al., 2000).

Depending on the indication, the use of one or the other tracer could be more beneficial. In specific neurologic or behavioral conditions, it may be more important either to visualize specific brain regions (such as the subcortical region which includes the limbic system) or to focus on brain perfusion or metabolism. From this perspective, it may be of importance to use the most suitable tracer. Future work should therefore explore the specific use of one of the tracers in research concerning specific neurological and behavioral conditions both in humans and animals.

F. Conclusion

The findings from the current study indicate that, while not entirely interchangeable, both tracers can be used for visualization of canine cerebral blood flow. Differences in regional distribution of both tracers suggest that a selection of either ^{99m}Tc -ECD or ^{99m}Tc -HMPAO

should be made and that direct comparisons between both tracers are not always recommended.

CHAPTER 2: The influence of a single dose of morphine on rCBF in dogs



Adapted from: Adriaens A, Peremans K, Waelbers T, Vandermeulen E, Duchateau L, Croubels S, Dobbeleir A, Audenaert K, Eersels J, Vermeire S, De Spiegeleer B, Polis I. The effect of morphine on canine regional cerebral blood flow measured by ^{99m}Tc -ECD SPECT. 2013c (submitted to Res Vet Sci).

A. Abstract

Regional cerebral blood flow (rCBF) can be used to assess brain activity patterns generated by drugs, in this case morphine, to gain insights in their mechanism of action. The aim of this study was to assess the effect of morphine on rCBF in dogs.

Methods: In a randomized cross-over experimental study, rCBF was estimated by means of ^{99m}Tc -ECD (Ethylcysteinate Dimer) single photon emission computed tomography (SPECT) in 8 dogs at baseline, at 30 minutes and at 120 minutes after a single bolus of morphine. The perfusion indices (PI) in the frontal, parietal, temporal and occipital cortex and in the subcortical and cerebellar region were obtained by semiquantification.

Results: A significantly decreased PI was found 30 min after morphine compared to baseline in the right frontal cortex ($P = 0.047$). The left parietal cortex and subcortical region showed a significantly increased PI 30 min after morphine compared to baseline ($P = 0.021$ and 0.006 respectively). No significant differences were noted for the other regions or at other time points.

Conclusions: A single bolus of morphine generated a different rCBF pattern at different time points. The cause for this difference should be investigated in future studies.

B. Introduction

Morphine, a mu opioid receptor agonist, is a frequently used potent analgesic in dogs (Pascoe, 2000a and 2000b). Its pharmacological effect depends on peripheral and supraspinal mu opioid receptor (MOR) activation (for review see Yaksh, 1997). Cerebral MORs are widely distributed throughout the brain and modulate the transmission and perception of painful stimuli (Mansour et al., 1995b).

Numerous studies have reported on different aspects of the influence of various opioids on cerebral hemodynamics, both in animal models and in man (Buchweitz et al., 1984; Hoffman et al., 1993; Firestone et al., 1996; Adler et al., 1997; Wagner et al., 2001; Nikolaishvili et al., 2004; Wagner et al., 2007; Zelaya et al., 2012). However, the effects of opioids on cerebral blood flow (CBF) remain unclear due to the variable results (Buchweitz et al., 1984; Hoffman et al., 1993; Firestone et al., 1996; Adler et al., 1997; Wagner et al., 2001; Nikolaishvili et al., 2004; Wagner et al., 2007; Zelaya et al., 2012). In dogs, only few have reported on the hemodynamic effects of morphine on the brain, usually reporting global cerebral blood flow changes with varying results (Takeshita et al., 1972; Matsumiya and Dohi, 1983; Milde et al., 1989; Rosen et al., 1989; Milde et al., 1990; Werner et al., 1991; Hoffman et al., 1993). These discrepancies are probably caused by differences in study design, imaging technique, species, opioid choice, dose and route of administration.

It is important to determine whether regional CBF (rCBF) differences occur in order to better understand how drugs influence cerebral activity and, therefore, to gain insight on their mechanism of action. Particularly in the case of mu opioid analgesics this is interesting since their side effects (dependence, tolerance, addiction) are thought to be mainly caused by supraspinal mechanisms in which the MOR plays a key role (Whistler, 2012).

Single photon emission computed tomography (SPECT) imaging using ^{99m}Tc -Ethylcysteinate Dimer (^{99m}Tc -ECD) is a valid method for rCBF evaluation in dogs (Peremans et al., 2001). As rCBF is correlated to regional brain activity (Gerrits et al., 1998), it can be used to study the effects of different drugs on the canine brain (Waelbers et al., 2011). The objective of the present study was to examine the effects of a single dose of morphine on rCBF in dogs. Since opiate action is not only dose, but also time dependent (Martin, 1983; Fuller and Stein, 1991), SPECT scans were performed at different time intervals after morphine administration.

C. Materials and methods

1. Animals

Eight female neutered Beagle dogs (age 4.22 ± 0.35 years, weight 8.58 ± 0.87 kg (mean \pm standard deviation, SD)) were used for this study. None of the dogs had a history of major disease or neurological disorder. The study was approved by the Ghent University Ethical Committee (EC 2011-130). All guidelines for animal welfare, imposed by the Ethical Committee, were respected.

2. Study Design

To investigate the influence of a single rapid bolus of morphine (0.5 mg/kg intravenously, Morphine Hydrochloride, Sterop, Belgium) on rCBF, all dogs were scanned 3 times in a randomized cross-over study design. The 3 SPECT scans consisted of a baseline scan (no morphine, condition 'Baseline'), a scan where ^{99m}Tc -ECD was injected 30 minutes after morphine (i.e. T30, condition 'MOR30') and a scan where ^{99m}Tc -ECD was injected 120 minutes after morphine (i.e. T120, condition 'MOR120'). All scans were performed following the same anesthetic and image acquisition protocol. Food, but not water, was

withheld for 12 hours before each scan. To determine morphine plasma and cerebrospinal fluid (CSF) concentrations 2 hours after morphine administration, 5 mL of blood were collected from the jugular vein (in a heparinized blood tube) and approximately 2 mL of CSF were collected by cisternal puncture from all dogs after the MOR30 scan. Blood was centrifuged at 3500 rpm for 5 minutes after which plasma was collected. All samples were kept at -20 °C until analysis was performed.

3. Tracer

To evaluate rCBF ^{99m}Tc -ECD (Neurolite[®], Bristol-Myers Squibb Medical Imaging), prepared according to the manufacturer's instructions, was used. ^{99m}Tc -ECD passes the blood-brain-barrier in its lipophilic form after which it is enzymatically transformed by intracellular esterases to a hydrophilic form that remains trapped in the cell. It rapidly reaches plateau values in the brain from 5 min onwards thus providing a fixed image of the rCBF practically at the moment of ^{99m}Tc -ECD injection. Optimal image acquisition is achieved between 15 and 40 min after tracer injection (Waelbers et al., 2012). ^{99m}Tc -ECD was administered intravenously: 747.91 ± 30.22 MBq, 789.16 ± 47.02 MBq and 751.61 ± 40.33 MBq in the Baseline, MOR30 and MOR120 condition, respectively.

4. Anesthetic Protocol

General anesthesia is required for SPECT imaging in animals. To avoid the influence of anesthetic drugs on rCBF, the administration of the anesthetics was done after the plateau for ^{99m}Tc -ECD was reached in the canine brain. The dogs were premedicated (intramuscular dexmedetomidine $375 \mu\text{g}/\text{m}^2$ body surface area, Dexdomitor[®], Orion Corporation, Espoo, Finland) 15 min after injection of ^{99m}Tc -ECD. Anesthesia was induced with propofol (1.74 ± 0.51 mg/kg intravenously, Propofol[®], Abbott Laboratories, Queenborough, United Kingdom)

and maintained with isoflurane (Isoflo[®], Abbott Laboratories) in oxygen. In order to limit the influence of the PaCO₂ on cerebral perfusion, dogs were mechanically ventilated (intermittent positive pressure ventilation) during anesthesia to preserve PaCO₂ within the physiologic range, i.e. end tidal CO₂ within 35-45 mmHg.

5. Image Acquisition

Image acquisition started 10 minutes after induction of anesthesia, i.e. 40 min after ^{99m}Tc-ECD injection. SPECT scans were performed using a triple head gamma camera (Triad Trionix, Twinsburg, OH, USA) equipped with ultrahigh resolution parallel hole collimators (tomographic resolution, 8 mm full width half maximum). For each acquisition, 120 projection images were obtained on a 128x128 matrix using a step-and-shoot mode (3° steps, 10 s per step), with a total scanning time of 20 minutes. During scanning, dogs were positioned in ventral recumbency with fixation of the head in a preformed cushion, to prevent individual positioning artifacts. Camera and table positioning were recorded to ensure optimal intraindividual comparison.

6. Image Processing

The images were reconstructed using filtered backprojection and post-filtering with a Butterworth filter (cut-off, 1.6 cycles/cm, order 5).

The emission data were fitted to a template using BRASS software (Brain Registration and Automated SPECT Semiquantification, Nuclear Diagnostics, Stockholm, Sweden) that allows scaling, rotating, and translating the acquired images in all 3 dimensions. This software was developed for humans but the template and regions were created in house for the use in dogs (Peremans et al., 2003b). The template provides the necessary anatomical references and a predefined region map for a volume-of-interest (VOI) approach. The following VOIs were included: right and left frontal, temporal, parietal and occipital cortices,

as well as the subcortical and cerebellar region. Perfusion indices (PI) for the different VOIs were calculated as the mean count per voxel in each VOI normalized to the mean total brain count per total voxel.

7. Morphine Concentrations

Plasma and CSF concentrations of morphine were determined using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. The quantification was based on the use of codeine as an internal standard (IS), which was added to the samples prior to analysis. Quality control (QC) and blank samples were analyzed together with each batch of incurred samples to check the extraction and LC-MS/MS procedure. All QC, blank and calibration curve samples were prepared in pooled drug-free dog plasma and drug-free artificial CSF (Tocris Bioscience, Abingdon, United Kingdom). This artificial CSF closely matches the electrolyte concentrations of CSF and final ion concentrations (in mM) are: Na 150; K 3.0; Ca 1.4; Mg 0.8; P 1.0; Cl 155. After addition of 25 μ L of the IS working solution of 10 μ g/mL to 250 μ L of the dog plasma and CSF samples, the pH was adjusted by addition of 500 μ L of a 0.67 M aqueous phosphate buffer of pH 7. After vortex mixing for 15 sec, the samples were put on an Oasis[®] MCX (mixed cation exchange) solid-phase extraction column (3 mL, 60 mg; Waters, Milford, MA, USA), previously conditioned with 2 mL of methanol and 2 mL of HPLC grade water. Subsequently, the column was washed with 2 mL of HPLC water, 1 mL of 0.1 M hydrochloric acid in water and 2 mL of methanol, respectively. Morphine and codeine were eluted with 2 mL of dichloromethane/2-propanol/ammonia (80/20/2, v/v/v). After evaporation at 50 °C under a gentle stream of nitrogen, the dry residue was dissolved in 100 μ L of 0.1% formic acid in HPLC water and filtered through a polyvinylidene fluoride 0.22 μ m filter (Millipore, Billerica, MA, USA). An aliquot of the extract (8 μ L) was injected onto the LC-MS/MS instrument. The HPLC system consisted of

an Acquity UPLC[®] instrument from Waters. The analysis of the extracts was performed on a BEH-C18 1.7 μ m column (2.1 x 50 mm; Waters), used in combination with a frit. A gradient elution with 0.1% formic acid in water (A) and acetonitrile (B) was performed (98% A/2% B, 0–2 min; 50% A/50% B, 2.1–3 min; 98% A/2% B, 3.1– 8 min). A Quattro Premier[®] XE MS instrument from Waters was used, equipped with an ESI (electrospray ionization) source, operated in the positive ion MS/MS mode. The following ion transition was monitored and used for quantification: m/z 286 > 165. The limit of detection of the method was calculated to be 1.27 ng/mL based on a signal-to-noise ratio of 3/1. The accuracy and precision were determined at 10.0 and 50.0 ng/mL in dog plasma ($n = 6$). The accuracy fell within the range of - 20% to +10%, and precision was also within the maximum RSD values as set by the EU (Anonymous, 2002). The limit of quantification (LOQ) was defined as the lowest concentration in dog plasma and CSF for which the method was validated with an accuracy and precision that fall within EU recommended ranges and was set at 5.0 ng/mL. Plasma and CSF concentrations below the LOQ were not included in the data analysis.

8. Statistical Analysis

To compare PI means between the three conditions, a mixed model was fitted with condition (Baseline, MOR30 and MOR120) as fixed effect and dog as random effect. Correlation between morphine plasma and CSF concentration was investigated by means of Kendall τ correlation coefficients.

Significance was set at $P < 0.05$. All data are presented as mean \pm SD.

D. Results

Table 1 depicts PIs for all VOIs in the different conditions. Mean PIs of the right frontal cortex, left parietal cortex and the subcortical region differed significantly between conditions. Pairwise comparison revealed that morphine administration only altered the rCBF

in those regions compared to the baseline condition at T30 ($P = 0.047$, 0.021 and 0.006 respectively). No significant differences were found when comparing Baseline to MOR120 or MOR30 to MOR120.

Table 1. Perfusion indices for the different conditions (Baseline, MOR30 and MOR120) in all brain regions in 8 dogs (mean \pm SD).

Brain region	Baseline	MOR30	MOR120	<i>P</i>
Left frontal	1.12 \pm 0.05	1.12 \pm 0.06	1.12 \pm 0.04	0.89
Right frontal	1.12 \pm 0.02	1.06 \pm 0.07	1.11 \pm 0.04	0.04*
Left temporal	0.86 \pm 0.02	0.90 \pm 0.07	0.89 \pm 0.05	0.23
Right temporal	0.85 \pm 0.05	0.85 \pm 0.04	0.90 \pm 0.05	0.09
Left parietal	1.12 \pm 0.07	1.21 \pm 0.06	1.19 \pm 0.07	0.02*
Right parietal	1.11 \pm 0.04	1.15 \pm 0.10	1.20 \pm 0.09	0.08
Left occipital	1.08 \pm 0.07	1.09 \pm 0.13	1.00 \pm 0.07	0.12
Right occipital	1.07 \pm 0.05	1.07 \pm 0.14	1.02 \pm 0.08	0.36
Subcortical	1.24 \pm 0.03	1.29 \pm 0.04	1.26 \pm 0.05	0.01*
Cerebellum	1.05 \pm 0.02	1.04 \pm 0.06	1.00 \pm 0.06	0.14

Data compared with mixed model. *Pairwise comparisons revealed a significant difference between Baseline and MOR30 in RF, LP and SC ($P = 0.047$, 0.021 and 0.006 respectively).

Two CSF samples had morphine concentrations below the LOQ and were not used for further analysis. Morphine plasma and CSF concentrations 2 hours after morphine administration were 13.50 ± 3.75 ng/mL and 17.52 ± 1.32 ng/mL, respectively (individual concentrations

are presented in Table 2). There was no significant correlation between morphine concentrations in plasma and CSF (Kendall τ correlation coefficient 0.43 and $P = 0.14$).

Table 2. Morphine concentrations (ng/mL) in plasma and CSF in 8 dogs 120 minutes after intravenous morphine administration at a dose of 0.5 mg/kg.

Dog	Plasma	CSF
1	16.9	17.6
2	12.6	19.1
3	18.0	18.5
4	10.1	15.7
5	7.2	< LOQ
6	15.3	18.0
7	16.3	< LOQ
8	11.6	16.2

LOQ: Limit of quantification of 5 ng/mL.

E. Discussion

The current study investigated changes in rCBF after morphine administration in dogs at two different time points. In non-painful dogs, a single dose of morphine induced rCBF changes in the frontoparietal cortex after 30 minutes compared to baseline values. We found a decreased blood flow in the frontal cortex and an increased blood flow in the parietal cortex. These brain regions are known to be involved in the complex - and not yet completely understood - mechanism of pain perception, i.e. the cognitive and affective-motivational aspects of nociception (for review see Ohara et al., 2005). Additionally, an increased CBF

was found in the subcortical region which includes structures important for pain modulation such as the thalamus and the periaqueductal gray (for review see Willis and Westlund, 1997). Alterations in rCBF reflect alterations in brain activity, i.e. increased brain activity requires increased blood supply and vice versa (Gerrits et al., 1998). It is therefore not surprising to find rCBF changes in regions involved in the modulation and perception of pain after the administration of a potent analgesic such as morphine. Furthermore, the present results are in line with a study in humans on the effects of opioids and provoked pain on rCBF (Wagner et al., 2007). In that study it was found that regions involved in the modulation of pain were activated, whereas regions that are involved in the perception of pain showed a decreased rCBF after opioid administration (Wagner et al., 2007). In dogs, the effects of opioids on global CBF have been investigated and decreased as well as increased global CBF have been reported (Matsumiya and Dohi, 1983; Milde et al., 1990; Werner et al., 1991). However, to our knowledge, the effect of opioids on regional CBF has not yet been studied in dogs.

Remarkably, we only found a significant difference in rCBF between MOR30 and Baseline and not between MOR120 and Baseline. In a study in dogs using remifentanyl, decreased blood flow and decreased EEG activity were described in the cortex, hippocampus and caudate nucleus (Hoffman et al., 1993). These changes recovered to baseline levels after 30 minutes (Hoffman et al., 1993). Since morphine is a longer acting opioid, it is possible that a comparable return to baseline brain activity occurs at a later time point.

The morphine plasma concentrations at T120 were 13.50 ± 3.75 ng/mL, these are consistent with concentrations needed to achieve 50% of maximal effect obtained in a pharmacodynamic study in dogs, i.e. 13.92 ± 2.39 ng/mL (KuKanich et al., 2005). In that study it was also reported that a significant analgesic level was present for only 2.8 ± 0.6 hours (KuKanich et al., 2005). In a clinical setting on the other hand, morphine is usually administered every 4 hours to maintain proper analgesia (Lamont and Matthews, 2007). In a

study in dogs on the pharmacokinetics of morphine, CSF concentrations at T120 were higher than plasma concentrations, as is the case in our study for most dogs and the range of CSF concentrations at T120 is also comparable to our results (Table 2) (Hug et al., 1981). It was additionally described that morphine appears to accumulate in CSF over time after systemic administration, since it has a longer halftime in CSF (121 ± 6 min) compared to plasma (75 ± 5 min) and the CSF-to-plasma concentration gradient increases progressively (Hug et al., 1981).

Taken together with our results, at T120 after morphine administration, we know that plasma and CSF concentrations are clinically relevant, however, no changes in blood flow – and thus brain activity – are detectable at that time point. This could possibly suggest that morphine exerts its effect at a different level than the brain at this time point, i.e. a peripheral action (spinal or at the periphery) rather than supraspinal action. This possibly insinuates a time-dependent localization of the analgesic effect of morphine, which would be interesting for further investigation in future studies.

As for the effects of opioids on cerebral hemodynamics in dogs, it is rather unlikely that changes in systemic cardiovascular parameters would cause regional alterations in CBF. Morphine at clinically relevant doses has limited influence on systemic cardiovascular parameters such as heart rate and blood pressure (Guedes et al., 2007; Maiante et al., 2009). Additionally, if proper ventilation is assured (opioids can cause respiratory depression), the effects of opioids on cerebral hemodynamics are limited (Armitage-Chan et al., 2007).

Several explanations for the alterations in rCBF caused by opioids have been suggested. The observed rCBF changes are most likely the result of changes in neuronal activity since MOR activation in itself causes increased energy demand, thus increasing blood flow. An effect of opioids on cerebral vasculature has also been proposed, either direct or indirect. Opioid receptors are found on cerebral vasculature (Peroutka et al., 1980) and opioids have

been found to be vasodilators (Wahl, 1985). However, this would imply that changes in rCBF are only related to MOR density, which is not always the case due to the widespread distribution of cerebral MORs (Trusk and Stein, 1987; Mansour et al., 1995b; Firestone et al., 1996; Khalili-Mahani et al., 2011) and the widespread influence of opioids on different neurotransmitter systems, including vasoactive systems such as nitric oxide, prostanoids or vasopressin (Armstead et al., 1990; Devine and Armstead, 1995; Armstead, 1996).

F. Conclusion

The present study found an altered rCBF pattern 30 minutes after morphine compared to baseline values. Pain is a complex phenomenon and unraveling the mode of action of analgesics such as opioids appears to be equally challenging, involving various cerebral regions. On top of this, the possibly time-dependent rCBF pattern found after morphine raises new questions on the correct interpretation of data and the comparison with other results.

Acknowledgments

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CHAPTER 3: The influence of prolonged morphine administration on rCBF in dogs



Adapted from: Adriaens A, Peremans K, Vandermeulen E, Waelbers T, Duchateau L, Croubels S, De Spiegeleer B, Polis I. The effect of prolonged administration of morphine on canine regional cerebral blood flow. 2013 (In preparation)

A. Abstract

The aim of the present study was to assess the influence of a prolonged morphine administration on the regional cerebral blood flow (rCBF) in dogs using functional brain imaging.

Methods: The rCBF was evaluated with ^{99m}Tc labeled hexamethylpropylene amine oxime (^{99m}Tc -HMPAO) single photon emission computed tomography (SPECT) in 8 dogs. All dogs were scanned at baseline and after 10 days of oral treatment with sustained release morphine sulphate in a randomized cross-over study. Perfusion was determined by normalizing regional perfusion (left and right frontal, parietal, temporal and occipital cortices and the subcortical and cerebellar regions) to total brain perfusion. Additionally, plasma and cerebrospinal fluid (CSF) morphine concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results: No significant changes in rCBF pattern could be detected. Plasma concentrations were consistently higher than CSF concentrations (ratio 5.74), but no correlation was found between both.

Conclusions: Despite clinically relevant plasma concentrations, the prolonged administration of morphine did not alter rCBF patterns in the canine brain.

B. Introduction

Morphine, a potent and frequently used analgesic in dogs and humans, is most commonly applied in cases of acute pain like perioperative pain management. Its use in chronic pain management in humans and animals is hindered due to the long-term side effects that can occur, such as tolerance, addiction and dependence. Functional imaging studies in man have mainly investigated cerebral blood flow alterations in subjects that had a history of substance abuse, including states of withdrawal, abstinence, craving and opioid maintenance therapy (van Dyck et al., 1994; Krystal et al., 1995; Danos et al., 1998; Rose et al., 1996; Schlaepfer et al., 1998; Pezawas et al., 2002; Guyer et al., 2007; Kosel et al., 2008; Suh et al., 2009). However, due to the great variation in study design, results are not consistent. Since animals are less likely to develop dependence and addiction, the most important side effect of long-term use of opioids in veterinary medicine, is the loss of therapeutic effect due to the development of tolerance.

Regional cerebral blood flow (rCBF) is known to be a measure for regional brain activity (Sokoloff et al., 1977; Gerrits et al., 1998). Therefore, by examining the rCBF changes produced by a drug with a supraspinal action, such as morphine, the rCBF pattern can be used to gain insights into its mode of action and to compare it with patterns generated by other drugs. To evaluate the rCBF in vivo in a non-invasive manner, single photon emission computed tomography (SPECT) with neuroperfusion tracers such as ^{99m}Tc -ECD (ethylcysteinate dimer) and ^{99m}Tc -HMPAO (hexamethylpropylene amine oxime) is a frequently used technique (Kapucu et al., 2009). The use of animal brain imaging studies provides advantages in drug research including the possibility to perform longitudinal brain imaging studies (less radioprotection issues, shorter life spans), ex vivo analysis of the brain after the imaging study, and assessment of new drugs prior to approval for human use

(review: Thompson and Bushnell, 2012). In this regard, both ^{99m}Tc -labelled tracers have been successfully used to investigate rCBF in dogs (Peremans et al., 2001 and 2003b; Martlé et al., 2009; Waelbers et al., 2011 and 2012; Adriaens et al., 2013a).

A recent study from our group investigated the effect of a single bolus of morphine on rCBF in dogs and found a time-dependent change in the rCBF pattern (Adriaens et al., 2013c (submitted)). Therefore, the present study aimed at determining whether a prolonged exposure to morphine at clinically relevant doses would induce similar or different changes in rCBF.

C. Materials and Methods

1. Animals

The study was approved by the Ghent University Ethical Committee (EC 2011-130). All guidelines for animal welfare, imposed by the Ethical Committee, were respected. Eight female neutered Beagle dogs (age 5.20 ± 0.34 years, weight 8.53 ± 0.87 kg (mean \pm standard deviation, SD)) were used for this study. None of the dogs had a history of major disease or neurological disorder.

2. Study Design

To evaluate the influence of a 10-day morphine treatment on rCBF, all dogs underwent 2 SPECT scans, i.e. Baseline and Morphine condition, in a randomized cross-over study (wash out period of 3 weeks). The Morphine condition scan was performed after a 10 days administration period of 20 mg oral sustained release morphine sulphate dosed twice daily (MS Contin[®], Mundipharma, Mechelen, Belgium), which corresponds to 2.37 ± 0.25 mg morphine/kg twice daily.

The dogs were monitored closely (respiratory rate, heart rate and quality, degree of sedation, nausea and emesis) for 3 hours after morphine administration and side effects were noted if observed.

Food, but not water, was withheld for 12 hours before each scan. To check whether the given morphine dosage was clinically relevant, morphine plasma concentrations were determined. For this, 5 mL of heparinized blood were collected from the jugular vein at 3 hours after the last morphine administration and before the Morphine scan. Blood was centrifuged at 3500 rpm for 5 min and plasma was collected. At the end of the Morphine scan, a cerebrospinal fluid (CSF) sample (2mL, collected by cisternal tap) was taken. All samples were kept at -20 °C until analyzed

3. Tracer

To evaluate rCBF ^{99m}Tc -HMPAO (Ceretec[®], GE Healthcare) was used and prepared according to the manufacturer's instructions. The ^{99m}Tc -HMPAO was administered intravenously: 361.58 ± 13.11 MBq and 339.75 ± 21.91 MBq in the Baseline and Morphine condition, respectively. ^{99m}Tc -HMPAO readily passes the blood-brain-barrier in its lipophilic form after which it is transformed to a hydrophilic form depending on the glutathione content and redox equilibrium in the cell (Jacquier-Sarlin et al., 1996a). This hydrophilic form then remains trapped in the cell.

4. Anesthetic Protocol

General anesthesia is required for SPECT imaging in animals. The dogs were premedicated with an intramuscular injection of dexmedetomidine at $375 \mu\text{g}/\text{m}^2$ body surface area, Dexdomitor[®], Orion Corporation, Espoo, Finland) once the plateau was reached for the rCBF tracer, i.e. 10 min after injection of ^{99m}Tc -HMPAO (Susskind et al., 1996). Anesthesia

was induced intravenously with propofol (1.69 ± 0.75 mg/kg intravenously, Propovet[®], Abbott Laboratories, Queenborough, United Kingdom) and maintained with isoflurane (Isoflo[®], Abbott Laboratories) in oxygen. In order to limit the influence of the PaCO₂ on cerebral perfusion, the dogs were mechanically ventilated (by intermittent positive pressure ventilation) during anesthesia to preserve PaCO₂ within the physiologic range, i.e. end tidal CO₂ within 35-45 mmHg.

5. Image Acquisition

Image acquisition started 10 minutes after induction of anesthesia, i.e. 35 min after ^{99m}Tc-HMPAO injection. SPECT scans were performed using a triple head gamma camera (Triad Trionix, Twinsburg, OH, USA) equipped with ultrahigh resolution parallel hole collimators (tomographic resolution, 8 mm full width half maximum). For each acquisition, 120 projection images were obtained on a 128x128 matrix using a step-and-shoot mode (3° steps, 15 s per step), with a total scanning time of 30 minutes. During scanning, the dogs were positioned in ventral recumbency with the head fixated in a preformed cushion, to prevent individual positioning artifacts. Camera and table positioning were recorded to ensure optimal intraindividual comparison.

6. Image Processing

The images were reconstructed using filtered backprojection and post-filtered using a Butterworth filter (cut-off 1.6 cycles/cm, order 5).

The emission data were fitted to a template using BRASS software (Brain Registration and Automated SPECT Semiquantification, Nuclear Diagnostics, Stockholm, Sweden) that allows scaling, rotating, and translating of the acquired images in all 3 dimensions. This software was developed for humans but the template and regions were created in house for the use in dogs (Peremans et al., 2003b). The template provides the necessary anatomical

references and a predefined region map for a volume-of-interest (VOI) approach. The following VOIs were included: right and left frontal, temporal, parietal and occipital cortices, as well as the subcortical and cerebellar region. Perfusion indices (PI) for the different VOIs for each dog were calculated as the count per voxel of the VOI normalized to the count per voxel of the whole brain.

7. Morphine Concentrations

Plasma and CSF concentrations of morphine were determined using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. The quantification was based on the use of codeine as an internal standard (IS), which was added to the samples prior to analysis. Quality control (QC) and blank samples were analyzed together with each batch of incurred samples to check the extraction and LC-MS/MS procedure. All QC, blank and calibration curve samples were prepared in pooled drug-free dog plasma and drug-free artificial CSF (Tocris Bioscience, Abingdon, United Kingdom). This artificial CSF closely matches the electrolyte concentrations of CSF and final ion concentrations (in mM) are: Na 150; K 3.0; Ca 1.4; Mg 0.8; P 1.0; Cl 155. After addition of 25 μ L of the IS working solution of 10 μ g/mL to 250 μ L of the dog plasma and CSF samples, the pH was adjusted by addition of 500 μ L of a 0.67 M aqueous phosphate buffer. After vortex mixing for 15 sec, the samples were put on an Oasis[®] MCX (mixed cation exchange) solid-phase extraction column (3 mL, 60 mg; Waters, Milford, MA, USA), previously conditioned with 2 mL of methanol and 2 mL of HPLC grade water. Subsequently, the column was washed with 2 mL of HPLC water, 1 mL of 0.1 M hydrochloric acid in water and 2 mL of methanol, respectively. Morphine and codeine were eluted with 2 mL of dichloromethane/2-propanol/ammonia (80/20/2, v/v/v). After evaporation at 50 °C under a gentle stream of nitrogen, the dry residue was dissolved in 100 μ L of 0.1% formic acid in HPLC water and filtered through a polyvinylidene fluoride

0.22 μm filter (Millipore, Billerica, MA, USA). An aliquot of the extract (8 μL) was injected onto the LC-MS/MS instrument. The HPLC system consisted of an Acquity UPLC[®] instrument from Waters. The analysis of the extracts was performed on a BEH-C18 1.7 μm column (2.1 x 50 mm; Waters), used in combination with a frit. A gradient elution with 0.1% formic acid in water (A) and acetonitrile (B) was performed (98% A/2% B, 0–2 min; 50% A/50% B, 2.1–3 min; 98% A/2% B, 3.1– 8 min). A Quattro Premier[®] XE MS instrument from Waters was used, equipped with an ESI (electrospray ionization) source, operated in the positive ion MS/MS mode. The following ion transition was monitored and used for quantification: m/z 286 > 165. The limit of detection of the method was calculated to be 1.27 ng/mL based on a signal-to-noise ratio of 3/1. The accuracy and precision were determined at 10.0 and 50.0 ng/mL in dog plasma ($n = 6$). The accuracy fell within the range of - 20% to +10%, and precision was also within the maximum RSD values as set by the EU (Anonymous, 2002). The limit of quantification (LOQ) was defined as the lowest concentration in dog plasma and CSF for which the method was validated with an accuracy and precision that fall within EU recommended ranges and was set at 5.0 ng/mL.

8. Statistical Analysis

The means of the obtained PIs from Baseline and Morphine were compared using a mixed model with treatment as fixed effect and dogs as random effects. Significance was set at $P < 0.05$. Correction for multiple comparisons was applied by means of Bonferroni correction. Correlation between morphine plasma and CSF concentrations was investigated by calculating the Kendall's τ correlation coefficient. Plasma to CSF ratio was calculated, as well as the mean of the ratios.

D. Results

The comparison of the mean PIs of the Baseline and Morphine conditions did not reveal statistically significant differences (Table 1). The observed differences for any of the regions were small.

Table 1. Perfusion indices (PI) for the Baseline and Morphine conditions measured with ^{99m}Tc -HMPAO in 8 dogs (mean \pm SD).

VOI	Baseline	Morphine	<i>P</i>
Left frontal	1.13 \pm 0.03	1.13 \pm 0.06	0.67
Right frontal	1.14 \pm 0.03	1.12 \pm 0.04	0.43
Left temporal	0.88 \pm 0.03	0.89 \pm 0.04	0.50
Right temporal	0.87 \pm 0.05	0.88 \pm 0.06	0.89
Left parietal	1.12 \pm 0.05	1.13 \pm 0.05	0.53
Right parietal	1.10 \pm 0.04	1.11 \pm 0.05	0.58
Left occipital	1.11 \pm 0.08	1.12 \pm 0.08	0.69
Right occipital	1.11 \pm 0.06	1.10 \pm 0.08	0.79
Subcortical	1.15 \pm 0.04	1.19 \pm 0.05	0.092
Cerebellum	1.12 \pm 0.08	1.13 \pm 0.05	0.93

Individual morphine plasma and CSF concentrations can be found in Table 2. Mean plasma concentrations were 47.60 ± 9.74 ng/mL and mean CSF concentrations were $9.53 \pm$

3.12 ng/mL. No correlation was found between the morphine plasma and CSF concentrations (Kendall τ correlation coefficient 0.43, $P = 0.14$). Additionally, the plasma/CSF ratios for each dog are represented in Table 2. The mean plasma/CSF ratio was 5.74.

Table 2. Individual morphine plasma and CSF concentrations (ng/mL) on the last day of morphine treatment (20 mg oral sustained release morphine sulphate twice daily for 10 days) in 8 dogs.

Dog	Plasma	CSF	Plasma/CSF Ratio
1	59.71	13.3	4.50
2	47.33	13.1	3.62
3	36.08	< LOQ (3.95)	-
4	51.97	8.5	6.14
5	59.31	6.8	8.75
6	35.79	5.9	6.07
7	51.56	9.6	5.35
8	39.05	< LOQ (4.36)	-

LOQ: Limit of quantification of 5 ng/mL

E. Discussion

A prolonged opioid treatment consisting of 10 days of morphine administration resulted in clinically relevant morphine concentration levels in plasma and CSF, but did not alter the rCBF pattern in non-painful dogs.

The dogs were given a 10-day morphine treatment during which the aim was not to induce tolerance, dependence or addiction. Rather, the duration of the treatment was chosen to create a longer period of treatment than what is usually administered during regular post-operative care. That way, the effects of a prolonged administration of morphine could be explored. Morphine can be used to treat chronic pain in small animals, but it is hindered by side effects and legal issues, therefore, the preference is usually given to non-steroidal anti-inflammatory drugs (Fox, 2010). In the present study, the 10-day morphine treatment was well tolerated by all dogs, supporting the possible use of morphine for longer periods. Additionally, a previous study from our group found that a similar morphine treatment induced changes in the 5-HT_{2A} receptor system, which can be beneficial for 5-HT_{2A} receptor-related diseases where prolonged treatment is often required (Adriaens et al., 2012b).

No difference in rCBF pattern could be detected in the present study. However, in the subcortical region, a small increase, though not significant, could be found in the morphine treated dogs. As mentioned, it is difficult to compare the present results to those in literature since most studies were conducted in man and studies involving prolonged administration of opioids generally focus on patients suffering from addiction, during abstinence, withdrawal craving or opioid maintenance therapy (Van Dyck et al., 1994; Krystal et al., 1995; Rose et al., 1996; Danos et al., 1998; Schlaepfer et al., 1998; Pezawas et al., 2002; Guyer et al., 2007; Kosel et al., 2008; Suh et al., 2009). Furthermore, due to the great variation in study design, it is often difficult to compare different, even contradictory, results. However, changes in rCBF – whether these were increases or decreases – were most consistently found in structures that are part of the limbic system (Krystal et al., 1995; Rose et al., 1996; Schlaepfer et al., 1998; Pezawas et al., 2002; Guyer et al., 2007; Suh et al., 2009). In this light, the present finding, although not statistically significant, of an increased rCBF in the subcortical region, which includes limbic structures such as the thalamus, is not entirely surprising.

The plasma and CSF morphine concentrations found are reported to be adequate to provide profound analgesia in dogs (Hug, 1981; KuKanich et al., 2005; Dohoo and Tasker, 1997). The variation in morphine plasma concentrations has also been described in other studies on oral morphine administration in dogs (Dohoo and Tasker, 1997) and no significant correlation between plasma and CSF concentrations was found. Additionally, morphine concentrations in plasma were consistently higher compared to those in CSF (plasma/CSF ratio 5.74).

In a previous study investigating the influence of a single bolus of morphine on rCBF in dogs, a possibly time-dependent effect of morphine was suggested. The alterations in rCBF in the cortical and subcortical regions found 30 min after morphine, returned to baseline values 2 hours after morphine administration. Even though the results of the latter study cannot be directly compared to those of the present study due to the use of a different radioligand (Adriaens et al., 2013a), it is striking that in both cases, despite the clinically relevant plasma concentrations of morphine, no significant alterations in rCBF were found. Interestingly, the same 10-day morphine treatment did produce changes in the cerebral serotonergic system in dogs, supporting that there is a supraspinal action, even though this is not reflected by blood flow changes (Adriaens et al., 2012b). This, however, remains to be further elucidated and should be of interest for future investigations on the complex mechanisms of opioid receptor internalization and desensitization. Additionally, the significance of the present studies and the relevance to pathophysiology of chronic opioid (ab)use also need further investigation.

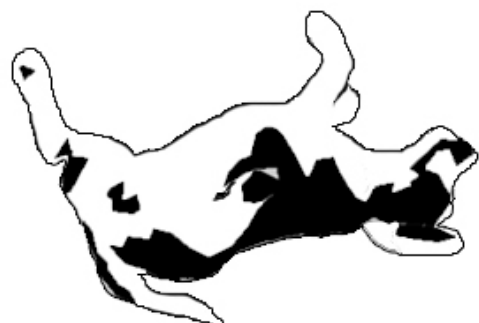
F. Conclusion

The prolonged administration of oral morphine in dogs resulted in clinically relevant plasma concentrations and did not alter the rCBF pattern measured by ^{99m}Tc -HMPAO SPECT. The use of oral morphine over a prolonged period was well tolerated and supports its use in perioperative pain management. The lack of significant rCBF alterations is in line with a previous report on the effect of morphine on rCBF in dogs.

Acknowledgements

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CHAPTER 4: The influence of a single dose of morphine on cerebral 5-HT_{2A} receptors in dogs



Adapted from: Adriaens A, Polis I, Vermeire S, Waelbers T, Duchateau L, Sys S, Van Dorpe S, Eersels J, De Spiegeleer B, Peremans K. The influence of morphine on cerebral 5-HT_{2A} receptor availability in dogs: a SPECT study. J Nucl Med. 2012a; 53(12), 1969-1973.

A. Abstract

The opioid and serotonergic systems are closely involved in pain processing and mood disorders. The aim of this study was to assess the influence of systemic morphine on cerebral 5-HT_{2A} receptor binding in dogs using single photon emission computed tomography (SPECT) with the 5-HT_{2A} radioligand ¹²³I-5I-R91150.

Methods: The 5-HT_{2A} receptor binding was estimated with (M) and without (C) morphine pre-treatment in eight dogs. The 5-HT_{2A} receptor binding indices (BIs) in the frontal, parietal, temporal and occipital cortex and in the subcortical region were obtained by semiquantification.

Results: A significantly decreased 5-HT_{2A} receptor BI was found in M for the right (M = 1.41 ± 0.06 , C = 1.53 ± 0.10) and left (M = 1.44 ± 0.08 , C = 1.55 ± 0.11) frontal cortices with $P = 0.012$ and $P = 0.040$, respectively. No significant differences were noted for the other regions.

Conclusions: Morphine decreased the frontocortical 5-HT_{2A} receptor availability, confirming an interaction between the 5-HT_{2A} and the opioid system. Whether this is caused by decreased receptor density due to direct internalization or the result of indirect actions, such as increased endogenous serotonin release, remains to be elucidated.

B. Introduction

It is well documented that there is a close interaction between the brain opioid and serotonin (5-hydroxytryptamine, 5-HT) neurotransmitter systems (Bardon and Ruckebusch, 1984; Tao and Auerbach, 1994; Zhao et al., 2007). Activation of the mu opioid receptor (MOR) system leads to 5-HTergic changes, including increased release of synaptic 5-HT. This was shown in several brain regions and in the spinal cord in various in vivo microdialysis studies (Bardon and Ruckebusch, 1984; Tao and Auerbach, 1994). Furthermore, pharmacological and behavioral studies pointed out that the 5-HTergic system is implicated in spinal pain transmission, modulation and control and particularly in the mediation of opioid analgesia (Bardin et al., 2000; Zhao et al., 2007). Morphine-induced analgesia is thought to rely in part on the indirect activation of descending inhibitory 5-HTergic pathways (Zhao et al., 2007). Consequently, antidepressants that inhibit 5-HT reuptake, such as fluvoxamine and fluoxetine, are also known to have analgesic properties, especially in chronic pain states (Honda et al., 2006; Verdu et al., 2008).

Beside their role in pain modulation (Kayser et al., 2007; Van Steenwinckel et al., 2008; Kupers et al., 2009), 5-HT_{2A} receptors gained interest over the last decades due to their involvement in several neurological disorders in humans, including anxiety (Nic Dhonnchadha et al., 2003), depression (Mintun et al., 2004), schizophrenia (Rasmussen et al., 2010) and obsessive-compulsive disorder (Rojas-Corrales et al., 2007). More specifically, atypical antipsychotic drugs acting as 5-HT_{2A} antagonists and inverse agonists were seen to be effective in reducing the symptoms of these diseases (Nic Dhonnchadha et al., 2003; Marek et al., 2003). Since the opioid system has been shown to be involved in several of these mood disorders as well (Rojas-Corrales et al., 2002), the interaction between MORs and 5-HT_{2A} receptors at an emotional and/or cognitive level gained interest, partly with

regard to common opioid side effects such as tolerance and addiction that are thought to have a serotonergic component as well. Consequently, several in vitro and agonist-antagonist behavioral studies were conducted in rodents, giving further evidence for this opioid-serotonin interaction (Marek and Aghajanian, 1998b; Marek, 2003; Rojas-Corrales et al., 2007; López-Giménez et al., 2008).

Functional brain imaging is a valuable tool for the investigation of neurotransmitter systems in vivo and is thus commonly used in research involving both neurological disorders and pain. The visualization of the brain 5-HT_{2A} receptor system in dogs with SPECT and the radioligand ¹²³I-5-I-R-91150 was proven to be efficacious in several studies. Studies regarding the role of the 5-HT_{2A} receptor system in canine anxiety, aggression and aging supported the application of a dog model as a suitable model for human disease (Peremans et al., 2002b; Peremans et al., 2003b; Vermeire et al., 2009).

The aim of the present study was to assess the influence of morphine on central 5-HT_{2A} receptors in dogs. More specifically, the possible change in 5-HT_{2A} receptor availability after an acute dose of morphine was investigated by means of functional brain imaging.

C. Materials and Methods

1. Animals

This study was approved by the Ghent University Ethical Committee (EC 2008-143). All guidelines for animal welfare, imposed by the Ethical Committee, were respected. To minimize gender, breed and age influence, eight female neutered Beagle dogs of approximately five years old were used for this study. The dogs weighted 13.04 ± 1.06 kg (mean \pm standard deviation, SD). None of the dogs had a history of previous major disease or neurological disorder.

2. Study Design

To assess the possible influence of morphine on the binding index (BI) of the 5-HT_{2A} receptor radioligand, BIs obtained by SPECT after morphine pre-treatment (M group) were compared to the BIs obtained without morphine pre-treatment (control or C group). Each dog was scanned twice (non-randomized) with a washout period between the C and M scan of at least two months. Images were acquired 90 minutes post radioligand injection, based on previous results concerning the optimal scanning time (Peremans et al., 2002a). The injected tracer activity was 14.73 ± 3.71 MBq/kg bodyweight (BW) for the C group and 15.42 ± 1.21 MBq/kg BW for the M group (mean \pm SD). In the M group, morphine pre-treatment (0.5 mg/kg BW, Morphine HCl, Sterop, Brussels, Belgium) was administered intravenously, injected manually over 1 minute, 30 minutes prior to radioligand injection. The used morphine dose is based on a clinically relevant dosage in dogs (Lamont and Matthews, 2007).

3. Tracer

¹²³I-5-I-R91150 is synthesized by electrophilic substitution on the 5-position of the methoxybenzamide group of R91150, followed by purification with high-performance liquid chromatography and was obtained from the Nuclear Medicine and PET research department of the VU University Medical Centre (Amsterdam, The Netherlands). The product had a radiochemical purity of more than 99 % and was sterile and pyrogen free. A specific activity of 370 GBq/ μ mol was obtained. The radioligand is a 5-HT_{2A} antagonist with high affinity ($K_d = 0.11$ nM) and selectivity for the 5-HT_{2A} receptor. The selectivity of the ligand for 5-HT_{2A} receptors with regard to other neurotransmitter receptors such as other 5-HT receptors, including 5-HT_{2C} and 5-HT_{1A}, dopamine receptors, adrenergic receptors and histamine

receptors is at least a factor 50. The tracer is displaceable with ketanserin, a 5-HT₂ antagonist (Catafau et al., 2006) and has been shown to be 5-HT_{2A} receptor-specific in dogs (Mertens et al., 1994; Peremans et al., 2002a).

4. Anesthetic Protocol

All dogs were sedated prior to general anesthesia with intramuscular dexmedetomidine 375 µg/m² body surface area. (Dexdomitor[®], Orion Corporation, Espoo, Finland). Anesthesia was induced with intravenous propofol (Propovet[®], Abbott Laboratories, Queenborough, United Kingdom), i.e. 2.22 ± 0.40 mg/kg BW for the C group and 1.55 ± 0.31 mg/kg BW for the M group (mean \pm SD). Anesthesia was maintained, after endotracheal intubation, with isoflurane (Isoflo[®], Abbott Laboratories, Queenborough, United Kingdom) (1.4% end tidal concentration) in oxygen using a rebreathing system. Acquisition started 10 minutes after induction of anesthesia. The dogs were mechanically ventilated by intermittent positive pressure ventilation to maintain eucapnia, i.e. end tidal CO₂ within 35-45 mmHg, in order to limit the influence of PaCO₂ on cerebral perfusion.

5. Image Acquisition

The dogs were positioned in ventral recumbency with fixation of the head in a preformed cushion, to prevent individual positioning artefacts. SPECT was performed with a triple head gamma camera (Triad Trionix, Twinsburg, OH, USA) equipped with ultrahigh resolution parallel hole collimators (Tomographic resolution, 8 mm full width half maximum). Images were acquired 90 minutes post injection of the radioligand. Total acquisition time was 30 minutes. For each acquisition, 90 projection images were obtained on a 128x128 matrix using a step-and-shoot mode (20 s/step, 4° steps). Camera and table positioning were recorded to ensure optimal 'intraindividual' comparison.

6. Image Processing

The radioligand data were reconstructed using the HERMES HybridRecon program (HERMES Medical Solutions, Stockholm, Sweden). The reconstruction is based on an ordered subset expectation maximization-based reconstruction algorithm with attenuation (using a CT transmission scan), collimator response and Monte Carlo-based scatter correction. The details of the reconstruction algorithm can be found in Sohlberg et al., 2008.

The ¹²³I-5-I-R91150 emission data were fitted to a template based on previously obtained cerebral perfusion data using multimodality software (version 5.0; Nuclear Diagnostic AB). This software displays images in a dual-window setting and allows for manual co-registration by providing tools for scaling, rotating, and translating images in all 3 dimensions. The perfusion data provide the necessary anatomical references using the volume-of-interest (VOI) approach. Following ROIs were included: right and left frontal, temporal, parietal and occipital cortices, as well as the subcortical and cerebellar region. The cerebellum, a region with low densities of 5-HT_{2A} receptors, is used as a reference region for non-specifically bound and free radioligand. Consequently, the measured radioactivity in the cortical regions represents the total activity, i.e. specific and non-specific bound and free radioligand. The BI was operationally calculated as ([counts per pixel in VOI] - [counts per pixel in cerebellum]) / (counts per pixel in cerebellum). This BI is proportional to the in vivo density of available receptors under pseudoequilibrium conditions (Pazos et al., 1987).

7. Statistical Analysis

The injected radioligand dose per kg BW and the injected propofol dose were compared between the two treatment groups using the *t*-test. Changes in 5-HT_{2A} binding with and without morphine pre-treatment were compared using the mixed model with ‘treatment’ and

‘ROI’ as categorical fixed effects and ‘dog’ as random effect. Left-right differences within groups were compared using a paired *t*-test. Data are presented as mean \pm SD. Level of significance was set at $P < 0.05$.

D. Results

No severe adverse reactions were seen after the morphine administration. All dogs were variably sedated. Some dogs showed mild nausea ($n = 4$) and panting ($n = 4$) and one dog had a short period of mild excitation. None of the dogs vomited.

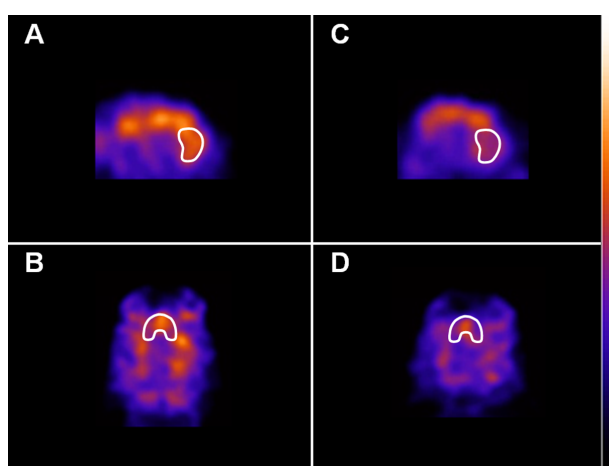
No significant differences were found between the two treatment groups for the injected radioligand dose per kg BW. A significant difference was found for the propofol dose per kg BW between groups, namely dogs in the M group required a lower propofol dose for induction ($P = 0.0013$).

Table 1 represents the 5-HT_{2A} BIs in the different VOIs for both groups. A significantly lower BI in the left ($P = 0.040$) and right ($P = 0.012$) frontal cortex was found in the dogs pre-treated with morphine compared to the control group (Table 1). The decreased 5-HT_{2A} receptor availability in the frontal cortex after morphine treatment can be visually appreciated in Figure 1.

Table 1 5-HT_{2A} binding indices with (M) and without (C) morphine pre-treatment (0.5 mg/kg IV) in 8 dogs (mean \pm SD).

VOI	C	M	P
Left frontal	1.55 \pm 0.11	1.44 \pm 0.08	0.040
Right frontal	1.52 \pm 0.10	1.41 \pm 0.06	0.012
Left temporal	1.40 \pm 0.10	1.36 \pm 0.08	0.343
Right temporal	1.41 \pm 0.09	1.37 \pm 0.08	0.344
Left parietal	1.41 \pm 0.07	1.37 \pm 0.08	0.191
Right parietal	1.39 \pm 0.11	1.37 \pm 0.12	0.585
Left occipital	1.35 \pm 0.10	1.31 \pm 0.08	0.080
Right occipital	1.37 \pm 0.09	1.33 \pm 0.10	0.447
Subcortical	1.16 \pm 0.10	1.09 \pm 0.08	0.134

Left-right comparison within groups showed no significant differences.

**Figure 1.** Sagittal (upper) and transversal (lower) ¹²³I-5I-R91150 SPECT images in one dog. Compared to the control images (A and B), 5-HT_{2A} receptor availability was decreased after morphine pre-treatment (C and D) in the frontal cortex (delineated region).

E. Discussion

A ¹²³I-5-I-R91150 SPECT study was conducted in dogs to examine the influence of a single intravenous bolus of morphine on 5-HT_{2A} binding and a decline in 5-HT_{2A} receptor availability was observed in the frontocortical region in dogs treated with morphine compared to the untreated group.

The BIs were only decreased significantly in the frontal cortex even though there was an overall decreased 5-HT_{2A} receptor binding after morphine treatment (Figure 1). Both MOR and 5-HT_{2A} receptors are widely distributed throughout the cortex (Tempel and Zukin, 1987; Blue et al., 1988). The frontal cortex, however, is known to be a region with both high MOR and 5-HT_{2A} receptor expression (Tempel and Zukin, 1987; Blue et al., 1988). The frontocortical region is involved in the control of the cognitive and emotional aspects of pain. MOR analgesics do not only exert their action through peripheral and spinal MOR activation, which alters the transmission of the noxious stimulus, on top of this, central MOR activation influences the perception of pain. Therefore, the frontal cortex is, not surprisingly, rich in MORs. In the frontal cortex 5-HT_{2A} receptors are well represented as well (Tempel and Zukin, 1987; Blue et al., 1988). Frontocortical 5-HT_{2A} receptors have been shown to play a role in several mood disorders, such as anxiety, aggression and depression not only in humans (Nic Dhonnchadha et al., 2003; Mintun et al., 2004), but also in dogs (Peremans et al., 2003b; Vermeire et al., 2009). These are mood disorders that often arise during opioid addiction and dependence and a 5-HT dysfunction is suggested to represent a main mechanism contributing to mood disorders in opiate abstinence (Goeldner et al., 2011). Additionally, 5-HT_{2A} receptor blockade is thought to restore some of the neurochemical modifications induced by long-term use of drugs of abuse, such as morphine (Lanteri et al., 2008).

The decreased 5-HT_{2A} receptor BIs found in the frontal cortex could be due to morphine-induced 5-HT release in the cortex, hence competing or interfering with 5-HT_{2A} radioligand binding. MOR agonists affect, next to their direct inhibitory action on neurons, several other neurotransmitter systems that contribute to opioid effects. One of these neurotransmitter systems is the 5-HT_{2A} system, greatly involved in mood regulation. It has been well established that the analgesic action of morphine relies in part on the activation of descending bulbospinal 5-HT_{2A} fibers. Supraspinal MOR activation by morphine leads to 5-HT release in the spinal cord (Bardon and Ruckebusch, 1984). Supraspinal MOR activation also causes increased 5-HT levels in several brain regions (Tao and Auerbach, 1994). In rodents for instance, systemic morphine increased extracellular 5-HT in the frontal cortex (Tao and Auerbach 1995). Another sequel of the increased synaptic 5-HT release could be downregulation and/or internalization of the 5-HT_{2A} receptor. However, the *in vitro* study from Van Oekelen and colleagues demonstrated that cell pre-treatment with 5-HT for 15 minutes and 48 hours did not provoke reduced 5-HT_{2A} receptor numbers (Van Oekelen et al., 2001). This pleads against down-regulation or internalization of the 5-HT_{2A} receptor by morphine invoked release of 5-HT.

Furthermore, it has been demonstrated that both receptors share a common intracellular pathway involving protein kinase C activation (López-Giménez et al., 2008). The latter study additionally reported that 5-HT_{2A} receptor activation resulted in a faster MOR desensitization, internalization and down-regulation (López-Giménez et al., 2008). By analogy, we might be tempted to interpret our results in a similar way, though the other way around, namely that MOR activation might increase 5-HT_{2A} receptor downregulation and/or internalization. An interaction between both receptors on glutamatergic neurons located in the neocortex has been suggested by the findings of Marek and colleagues (Marek and Aghajanian, 1998b).

Both receptors appear to regulate glutamate release presynaptically: 5-HT_{2A} receptor activation promotes glutamate release, whereas MOR activation inhibits this enhanced glutamate release (Marek and Aghajanian, 1998b). The glutamate release induced by 5-HT_{2A} receptor activation is thought to mediate a typical behavioral pattern of head shaking and twitching, which is seen after frontocortical 5-HT_{2A} receptor activation (Willins and Meltzer, 1997). Accordingly, this behavior was attenuated after MOR agonist (buprenorphine, fentanyl) administration (Marek, 2003).

It must be stated that this might not be the only cause for the lower BIs found. SPECT imaging is an elegant technique to quantify neurotransmitter systems in vivo, albeit a degree of caution is warranted when interpreting the images since the present findings may reflect other types of interactions as well. Direct interactions involving direct binding of morphine to the 5-HT_{2A} receptor may be another possibility, since studies exist that reported direct interactions between opioids and 5-HT₂ receptors. For instance, morphine was shown to interact with human 5-HT_{2A} receptors (Wittmann et al., 2006), and fentanyl shows, albeit low, affinity for 5-HT_{1A} receptors (Tao et al., 2003). To our knowledge however, no such interaction has been described for morphine and 5-HT_{2A} receptors.

In a recent study comparing the effects of morphine on serotonergic neurons in midbrain areas in the presence or absence of acute noxious stimulation, it was found that those serotonergic neurons responded differently to morphine when pain was present, i.e. they showed a higher neuronal activity (Bajic and Commons, 2010). The influence of morphine on the 5-HT_{2A} receptor availability might be different in the presence of acute noxious stimulation.

The interference of anesthetics with radioligand binding is another consideration that needs to be taken into account (Giron, 2009). However, both groups underwent the same anesthetic protocol, with the exception of the propofol dose, thus eliminating interference of

anesthetics with radioligand binding as a possible confounding factor. Several studies on the influence of different anesthetic agents on the central serotonergic system have shown that isoflurane reduces 5-HT release (Mukaida et al., 2007), whereas propofol increases synaptic 5-HT levels by blocking the 5-HT transporter, SERT (Zhao and Sun, 2008). In the present study, the C group was administered more propofol compared to the M group. However, recent findings from our research group (unpublished data) suggested no difference in 5-HT_{2A} receptor binding between low and high propofol dosage, implying that the difference in propofol dosage between the two treatment groups in this study would be of minor importance. Additionally, we found that morphine did not influence the regional cerebral blood flow in dogs (Adriaens et al., 2013c), therefore this cannot account for the found changes in 5-HT_{2A} receptor BIs.

F. Conclusion

Our findings support the existence of an interaction between morphine and 5-HT_{2A} receptor binding in the canine frontal cortex. In the absence of pain and mood disorders, morphine decreased the 5-HT_{2A} receptor availability. Future studies investigating the exact mechanism of this interaction would be of great interest, including the influence of morphine on SERT. Overall the present study supports the possible beneficial effect of 5-HT_{2A} receptor drugs in the prevention of common opiate side effects such as dependence and addiction, as well as the use of opioids in mood disorders.

CHAPTER 5: The influence of prolonged morphine administration on cerebral 5-HT_{2A} receptors in dogs



Adapted from: Adriaens A, Polis I, Vermeire S, Waelbers T, Croubels S, Duchateau L, Van Dorpe S, Eersels J, De Spiegeleer B, Peremans K. The effect of prolonged exposure to morphine on canine cerebral 5-HT_{2A} receptors measured with ¹²³I-R91150 SPECT. 2013b (submitted to Eur Neuropsychopharmacol).

A. Abstract

Drugs acting on the serotonergic 2A (5-HT_{2A}) receptor, as well as opioids have been reported as augmenting agents in depression therapy. The present study examined the effect of prolonged opioid treatment on cerebral 5-HT_{2A} receptors.

Methods: Brain 5-HT_{2A} receptor availability was estimated in seven healthy five-year-old female neutered Beagle dogs pre and post 10-day morphine treatment (oral sustained release morphine 20 mg twice daily for ten days) with ¹²³I-R-91150, a 5-HT_{2A} selective radioligand, and SPECT. 5-HT_{2A} receptor binding indices (BI) for the frontal, parietal, temporal and occipital cortex and the subcortical region were calculated. Data were analyzed by mixed model with treatment as fixed effect and dog as random effect.

Results: Morphine treatment significantly ($P \leq 0.05$) lowered 5-HT_{2A} BIs in the right and left frontal cortex, the right and left temporal cortex, the right and left parietal cortex, and the subcortical region.

Conclusion: The decreased cerebral 5-HT_{2A} receptor availability following prolonged morphine exposure suggests an interaction between the opioid and serotonergic system, the exact nature of this interaction remains to be elucidated.

B. Introduction

Enhancing the synaptic availability of serotonin (5-HT) is one of the critical components of pharmacological treatment of depression and most antidepressant drugs act on the inhibition of serotonin re-uptake. As a result, selective serotonin reuptake inhibitors (SSRIs) are commonly used for the treatment of depression. However, long-term remission of symptoms cannot always adequately be maintained with the currently available antidepressant drugs (Nemeroff, 2007). Moreover, it takes several days to weeks before these antidepressants exert their full clinical effect. This lag-time in onset suggests that downstream neuronal adaptations are more likely to be responsible for the therapeutic effects of antidepressants, rather than the immediate synaptic 5-HT elevation itself (Manji et al., 2000).

The last decade, more attention has been paid to the down-stream molecular effects occurring during depression and antidepressant treatment. It has been suggested that abnormalities in monoamine receptors and a relative deficiency of monoamines may lead to depression (Stahl, 2008). In depressed patients, adaptive upregulation of postsynaptic receptors, such as the serotonergic 2A (5-HT_{2A}) receptor, has been observed (Cahir et al., 2007; Shelton et al., 2009). Furthermore, 5-HT_{2A} receptor expression has been shown to be altered after SSRI treatment in humans and dogs (Gray and Roth, 2001; Peremans et al., 2005). Atypical antipsychotics with 5-HT_{2A} receptor antagonist properties have been shown to augment the therapeutic effect of SSRIs (Ostroff and Nelson, 1999; Marek et al., 2003; Philip et al., 2008; Nelson and Papakostas, 2009). Therefore, the postsynaptic 5-HT_{2A} receptor is thought to be a possible target for antidepressant therapy.

Mu opioid receptor (MOR) agonists have been reported to increase serotonin levels in the brain (Tao and Auerbach, 1994) and in view of this, the use of opioids as additional antidepressant therapeutics has been investigated. Indeed, biochemical and behavioral studies

suggest the involvement of the opioid system in the mechanism of action of certain antidepressants (Fichna et al., 2007a). Additionally, opioids have been found to have antidepressant-like properties (Bodkin et al., 1995; Tejedor-Real et al., 1995; Besson et al., 1996; Stoll and Rueter, 1999). However, the exact potential of opioids as antidepressant agents remains a matter of discussion.

In previous studies we have shown that the dog can be used as a suitable model for humans for single photon emission computed tomography imaging (SPECT) of the 5-HT_{2A} receptors using the radioligand ¹²³I-R91150 (Peremans et al., 2003b; Vermeire et al., 2009). Additionally, we have previously demonstrated that a single dose of morphine lowered the 5-HT_{2A} receptor availability in the canine frontal cortex (Adriaens et al., 2012a).

Since both opioids, like morphine, and 5-HT_{2A} receptors appear to be involved in similar biological processes, the aim of the present study was to assess the influence of prolonged administration of morphine on the cerebral 5-HT_{2A} receptors in dogs.

C. Materials and Methods

1. Animals

Seven healthy Beagle dogs (neutered females), ranging in weight from 6.4 kg to 7.4 kg, and age from 3 to 4 years old were used in this study. None of the dogs had a history of previous major disease or neurological disorder. All procedures and protocols were approved by the Ghent University Ethical Committee (EC2010/152).

2. Study Design

Each dog underwent two SPECT scans. The first scan consisted of a baseline scan (pre-morphine condition) to which data from the second scan (post-morphine condition) were compared. Post-morphine scans were performed after an opioid treatment that consisted of an oral sustained release formulation of morphine (20 mg morphine sulphate, MS Contin[®],

Mundipharma, Mechelen, Belgium) administered orally twice daily for 10 days, which is comparable to a dosage of 2.86 ± 0.16 mg/kg twice daily. The dogs were monitored closely (respiratory rate, heart rate and quality, degree of sedation, nausea and emesis) for 3 hours after morphine administration and side effects were noted if observed.

Food, but not water, was withheld for 12 hours before each scan. To check whether the given morphine dosage was clinically relevant, morphine plasma concentrations were determined. For this, 5 mL of blood were collected from the jugular vein and placed in a heparinized blood tube the last day of treatment 3 hours after the last morphine administration. Blood was centrifuged at 3500 rpm for 5 minutes after which plasma was collected and kept at -20 °C until analysis was performed.

3. Tracer

¹²³I-R91150 is synthesized by electrophilic radioiodination on the 5-position of the methoxybenzamide group of R91150 (Eersels et al., 2005), followed by purification with high-performance liquid chromatography and was obtained from the Nuclear Medicine and PET research department of Nuclear Medicine and PET research of the VU University Medical Centre (Amsterdam, The Netherlands). The product had a radiochemical purity of more than 99 % and was sterile and pyrogen free. The administered radioligand had a specific activity of 370 GBq/μmol. The radioligand is a 5-HT_{2A} antagonist with high affinity ($K_d = 0.11$ nM) and selectivity for the 5-HT_{2A} receptor. The selectivity of the ligand for 5-HT_{2A} receptors with regard to other neurotransmitter receptors such as other 5-HT receptors, including 5-HT_{2C} and 5-HT_{1A}, dopamine receptors, adrenergic receptors and histamine receptors is at least a factor 50. The tracer is displaceable with ketanserin, a 5-HT₂ antagonist (Catafau et al., 2006) and has been previously shown to be suitable for the use in dogs (Mertens et al., 1994; Peremans et al., 2002a).

4. Anesthetic Protocol

All dogs were sedated prior to general anesthesia with intramuscular dexmedetomidine 375 µg/m² body surface area (Dexdomitor[®], Orion Corporation, Espoo, Finland). After placing a 22-gauge intravenous (IV) catheter in the cephalic vein, anesthesia was induced with IV propofol (Propofol[®], Abbott Laboratories, Queenborough, United Kingdom), 2.14 ± 0.56 mg/kg for the pre-morphine condition and 1.86 ± 0.38 mg/kg for the post-morphine condition (mean \pm SD). Anesthesia was maintained, after endotracheal intubation, with isoflurane (Isoflo[®], Abbott Laboratories) (1.4% end tidal concentration) in oxygen using a rebreathing system. Acquisition started 10 minutes after induction of anesthesia. The dogs were mechanically ventilated by intermittent positive pressure ventilation to maintain eucapnia, i.e. end tidal CO₂ within 35-45 mmHg, in order to limit the influence of the PaCO₂ on cerebral perfusion.

5. Image Acquisition

The dogs were positioned in ventral recumbency with fixation of the head in a preformed cushion, to prevent individual positioning artifacts. SPECT was performed with a triple head gamma camera (Triad Trionix, Twinsburg, OH, USA) equipped with ultrahigh resolution parallel hole collimators (FWHM of 8 mm). Images were acquired 90 minutes post injection of the radioligand, this is the moment at which pseudoequilibrium is present in dogs (21). Total acquisition time was 30 minutes. For each acquisition, 90 projection images were obtained on a 128x128 matrix using a step-and-shoot mode (20 s/step, 4° steps). Camera and table positioning were recorded to ensure optimal 'intraindividual' comparison.

6. Image Processing

The radioligand data were reconstructed using the HERMES HybridRecon program (HERMES Medical Solutions, Stockholm, Sweden). The reconstruction is based on an

ordered subset expectation maximization-based reconstruction algorithm with attenuation (using a CT transmission scan), collimator response and Monte Carlo-based scatter correction. The details of the reconstruction algorithm can be found in Sohlberg et al., 2008. Reconstructed images were post-filtered using a Butterworth filter (cut-off, 1.6 cycles/cm, order 5).

The ¹²³I-R91150 emission data were fitted to a template, that is based on previously obtained cerebral perfusion data, using multimodality software (version 5.0; Nuclear Diagnostic AB) (Peremans et al., 2003a). This software displays images in a dual-window setting and allows for manual co-registration by providing tools for scaling, rotating, and translating images in all 3 dimensions. The following volumes of interest (VOIs) were included: right and left frontal, temporal, parietal and occipital cortices, as well as the subcortical and cerebellar region. The cerebellum, a region with low densities of 5-HT_{2A} receptors, is used as a reference region for non-specifically bound and free radioligand. Consequently, the measured radioactivity in the cortical regions represents the total activity, i.e. specific and non-specific bound and free radioligand. The binding index (BI) was calculated as $([\text{counts per pixel in VOI}] - [\text{counts per pixel in cerebellum}]) / (\text{counts per pixel in cerebellum})$. This BI is proportional to the in vivo density of available receptors under pseudoequilibrium conditions (Pazos et al., 1987).

7. Morphine Plasma Concentrations

Plasma concentrations of morphine were determined using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. The quantification was based on the use of codeine as an internal standard (IS), which was added to the homogenized plasma samples prior to analysis. Quality control (QC) and blank samples were analyzed together with each batch of incurred samples to check the extraction and LCMS/MS

procedure. All QC, blank and calibration curve samples were prepared in pooled drug-free dog plasma. After addition of 25 μ L of the IS working solution of 10 μ g/mL to 250 μ L of the dog plasma samples, the pH was adjusted by addition of 500 μ L of a 0.67 M aqueous phosphate buffer. After vortex mixing for 15 sec, the samples were put on an Oasis[®] MCX (mixed cation exchange) solid-phase extraction column (3 mL, 60 mg; Waters, Milford, MA, USA), previously conditioned with 2 mL of methanol and 2 mL of HPLC grade water. Subsequently, the column was washed with 2 mL of HPLC water, 1 mL of 0.1 M hydrochloric acid in water and 2 mL of methanol, respectively. Morphine and codeine were eluted with 2 mL of dichloromethane/2-propanol/ammonia (80/20/2, v/v/v). After evaporation at 50 °C under a gentle stream of nitrogen, the dry residue was dissolved in 100 μ L of 0.1% formic acid in HPLC water and filtered through a polyvinylidene fluoride 0.22 μ m filter (Millipore, Billerica, MA, USA). An aliquot of the extract (8 μ L) was injected onto the LC-MS/MS instrument. The HPLC system consisted of an Acquity UPLC[®] instrument from Waters. The analysis of the extracts was performed on a BEH-C18 1.7 μ m column (2.1 x 50 mm; Waters), used in combination with a frit. A gradient elution with 0.1% formic acid in water (A) and acetonitrile (B) was performed (98% A/2% B, 0–2 min; 50% A/50% B, 2.1–3 min; 98% A/2% B, 3.1–8 min). A Quattro Premier[®] XE MS instrument from Waters was used, equipped with an ESI (electrospray ionization) source, operated in the positive ion MS/MS mode. The following ion transition was monitored and used for quantification: m/z 286 > 165. The limit of detection of the method was calculated to be 1.27 ng/mL based on a signal-to-noise ratio of 3/1. The accuracy and precision were determined at 10.0 and 50.0 ng/mL in dog plasma (n = 6). The accuracy fell within the range of - 20% to +10%, and precision was also within the maximum RSD values as set by the EU (Anonymous, 2002). The limit of quantification (LOQ) was defined as the lowest concentration in dog plasma for which the method was validated with an accuracy and precision that fall within EU

recommended ranges and was set at 5.0 ng/mL. Plasma concentrations below the LOQ were not included in the data analysis.

8. Statistical Analysis

Changes in 5-HT_{2A} receptor binding before and after morphine treatment were compared using the mixed model with ‘treatment’ as fixed effect and ‘dog’ as random effect. Data are presented as mean \pm standard deviation (SD). Level of significance was set at $P < 0.05$ and $P < 0.0056$ when correcting for multiple comparisons (Bonferroni).

D. Results

The 10-day morphine treatment was well tolerated by the dogs. At any point during treatment we recorded the following side effects: nausea (number of dogs, $n = 1$), vomiting ($n = 1$).

Table 1 represents the 5-HT_{2A} receptor BIs in the different VOIs for both conditions. Significantly lower ($P < 0.05$) BI values were found in the right and left frontal cortex, the right and left temporal cortex, the right and left parietal cortex and the subcortical region for the post-morphine condition compared to the pre-morphine condition (Table 1). After correction for multiple comparisons 5-HT_{2A} receptor BIs were still significantly ($P < 0.0056$) decreased in the left frontal cortex and the right parietal cortex after morphine treatment. The decreased 5-HT_{2A} receptor density in the post-morphine condition of one dog can be visualized in Figure 1.

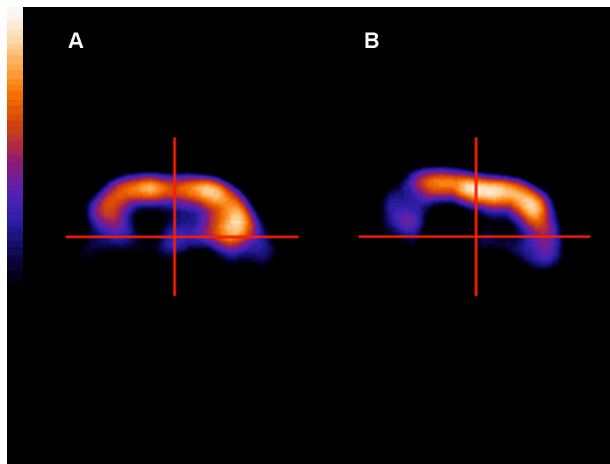


Figure 1. Sagittal ^{123}I -R91150 SPECT images in one dog. Compared to the pre-morphine images (A), 5-HT_{2A} receptor availability was decreased after 10 days of morphine treatment (B).

Table 1. 5-HT_{2A} receptor binding indices before and after chronic morphine treatment (20 mg/kg oral sustained release morphine sulphate, twice daily for 10 days) (mean \pm SD).

VOI	Pre-morphine	Post-morphine	<i>P</i>
Left frontal	1.60 \pm 0.15	1.40 \pm 0.11	0.004*
Right frontal	1.61 \pm 0.19	1.45 \pm 0.10	0.038
Left temporal	1.48 \pm 0.17	1.34 \pm 0.10	0.019
Right temporal	1.51 \pm 0.15	1.35 \pm 0.10	0.026
Left parietal	1.55 \pm 0.18	1.36 \pm 0.12	0.009
Right parietal	1.56 \pm 0.17	1.37 \pm 0.18	0.004*
Left occipital	1.46 \pm 0.20	1.36 \pm 0.18	0.175
Right occipital	1.47 \pm 0.17	1.38 \pm 0.15	0.237
Subcortical	1.33 \pm 0.18	1.19 \pm 0.13	0.012

* $P < 0.0056$ (Bonferroni correction).

The morphine plasma concentration from the post-morphine condition on day 10, 3 hours after the last morphine administration was found to be 29.8 ± 7.8 ng/ml. Individual concentrations are shown in Table 2.

Table 2. Morphine plasma concentrations (ng/mL) on the last day of treatment, 3 hours after intake (20 mg/kg oral sustained release morphine sulphate, twice daily for 10 days).

Dog	Plasma
1	25.3
2	37.3
3	19.6
4	35.3
5	24.6
6	40.4
7	25.8

E. Discussion

5-HT_{2A} receptors have been thought to be involved in the therapeutic effect of certain antidepressant drugs. Since opioids potentially have antidepressant effects, the present study investigated whether a prolonged morphine administration would have an influence on the 5-HT_{2A} receptor density. It was found that a 10-day morphine treatment decreased the 5-

HT_{2A} receptor availability in the majority of the canine cerebral regions measured with SPECT by means of the 5-HT_{2A} receptor radioligand ¹²³I-R91150.

Traditionally, antidepressant therapy is based on increasing monoamine levels in the brain, 5-HT in particular, resulting in adaptive down-regulation of post-synaptic receptors, such as the 5-HT_{2A} receptor (Klimek et al., 1994; Gray and Roth, 2001; Meyer et al., 2001; Peremans et al., 2005). It has therefore been suggested that the effect of antidepressants on the 5-HT_{2A} receptor contributes to their therapeutic effect. This hypothesis is confirmed by several studies on the use of atypical antipsychotics with 5-HT_{2A} receptor antagonist properties as antidepressant therapy augmentation agents (Philip et al., 2008; Nelson and Papakostas, 2009). The results of our study demonstrate a decreased 5-HT_{2A} receptor BI after 10 days of morphine treatment. These results are in line with the decreased 5-HT_{2A} receptor density that was reported in dogs after 6-weeks of SSRI treatment, together with reduced impulsive aggression (Peremans et al., 2005). This behavioral improvement associated with the 5-HT_{2A} receptor down-regulation underlines the clinical relevance of the observed findings.

Morphine is a potent mu opioid receptor (MOR) agonist and since MORs and their endogenous ligands, such as endomorphins, are localized in brain regions involved in depression, it has been suggested that the endogenous opioid system could play a role in the pathogenesis of depression (Fichna et al., 2005a). For instance, the antidepressant-like effects of endomorphins in rodents was found to rely on their action on MORs (Fichna et al., 2007b). MOR agonists have been shown to produce a significant antidepressant effect not only in rodent models of depression, but also in humans suffering from treatment-refractory depression (Tejedor-Real et al., 1995; Bodkin et al., 1998; Stoll and Reuter 1999).

The interaction between the serotonergic and opioid system has been extensively studied not only in the search towards new antidepressants but even more in the field of analgesia.

Modulation of the serotonergic system (e.g. by re-uptake inhibition) has been investigated in search for augmentation of the analgesic efficacy of opioids and the use of antidepressants is one of the well-established treatment options for chronic pain (Vadalouca et al., 2006; Banks et al., 2010). In this light, the 5-HT_{2A} receptor is an interesting subject. Frontocortical 5-HT_{2A} receptors are thought to be involved in several mood disorders, such as anxiety, aggression and depression both in humans and dogs (Nic Dhonnchadha et al., 2003; Peremans et al., 2003; Mintun et al., 2004; Vermeire et al., 2009). Such mood disorders often occur during opioid addiction and dependence as well, and a 5-HT dysfunction is suggested to represent a main mechanism contributing to mood disorders in opiate abstinence (Goeldner et al., 2011). Additionally, blocking 5-HT_{2A} receptors is thought to restore some of the neurochemical modifications induced by the long-term use of drugs of abuse, such as morphine (Lanteri et al., 2008). Even though our animals were not physically dependent on morphine, our results are in line with the previously mentioned findings that not only there is a serotonergic component in chronic opioid side effects, but that, more specifically, the 5-HT_{2A} receptor might be involved as well. Hence, the present findings could be of interest for future studies on opioid side effects.

The morphine plasma concentrations determined in this study correspond to the clinically relevant concentrations found in literature (Dohoo, 1997). To achieve physical dependence or tolerance to morphine, higher dosages are needed (Yoshimura et al., 1993). However, this was not the goal of this study. Moreover, the use of opioids as potential antidepressant agents would be severely hindered due to side effects if high doses were required.

The exact mechanism by which morphine decreases the 5-HT_{2A} receptor availability in our study, remains to be elucidated. SPECT imaging is an excellent technique for visualizing and semi-quantifying neurotransmitter systems *in vivo*, however, it cannot provide tangible answers on the exact mechanisms that underlie the alterations in receptor binding. Since

morphine was administered during 10 days, an increased 5-HT tone could account for an adaptive down-regulation of the 5-HT_{2A} receptors. An acute surge (15 minutes to 48 hours) in 5-HT could not provoke reduced 5-HT_{2A} receptor numbers (Van Oekelen et al., 2001), however, chronic SSRI treatment in humans resulted in decreased 5-HT_{2A} receptor density (Meyer et al., 2001). This was also seen in dogs, where 6 weeks of inhibition of serotonin reuptake resulted in decreased cortical 5-HT_{2A} receptor binding (Peremans et al., 2005). Albeit 5-HT release was found to be reduced in morphine dependent rodents (Jolas et al., 2000), as stated previously, the dogs used in the present study were not considered to be dependent on morphine. This possibly implies a different alteration of the serotonergic system during opioid dependency compared to prolonged low-dose opioid use.

Another possible explanation for the morphine-induced decrease in 5-HT_{2A} receptor binding could be an interaction between the MOR and 5-HT_{2A} receptor. Both receptors are widely distributed in the brain (Tempel and Zukin, 1987; Blue et al., 1988) and it has been shown that 5-HT_{2A} receptor activation influences MOR internalization, probably through a common intracellular pathway (López-Giménez et al., 2008). Thus it might be possible as well that MOR activation influences 5-HT_{2A} receptor internalization and down-regulation. Competition between the radioligand and morphine for the 5-HT_{2A} receptor could also occur, however, the affinity of morphine for the 5-HT_{2A} receptor is rather low (Leysen et al., 1982). Possible allosteric changes of the 5-HT_{2A} receptors due to morphine binding would also be an interesting subject for future studies.

F. Conclusion

Prolonged exposure to morphine decreased the 5-HT_{2A} receptor availability in a large part of the canine cortex. This finding gives further evidence for the existence of an

interaction between the opioid and 5-HT_{2A} receptor system and encourages further research on the possible beneficial effects of opioids in the treatment of mood disorders.

Acknowledgements

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GENERAL DISCUSSION



In the following section, the results of the present doctoral thesis (Figure 1) will be discussed and compared to the existing literature. Additionally, study limitations and future prospects will be considered.

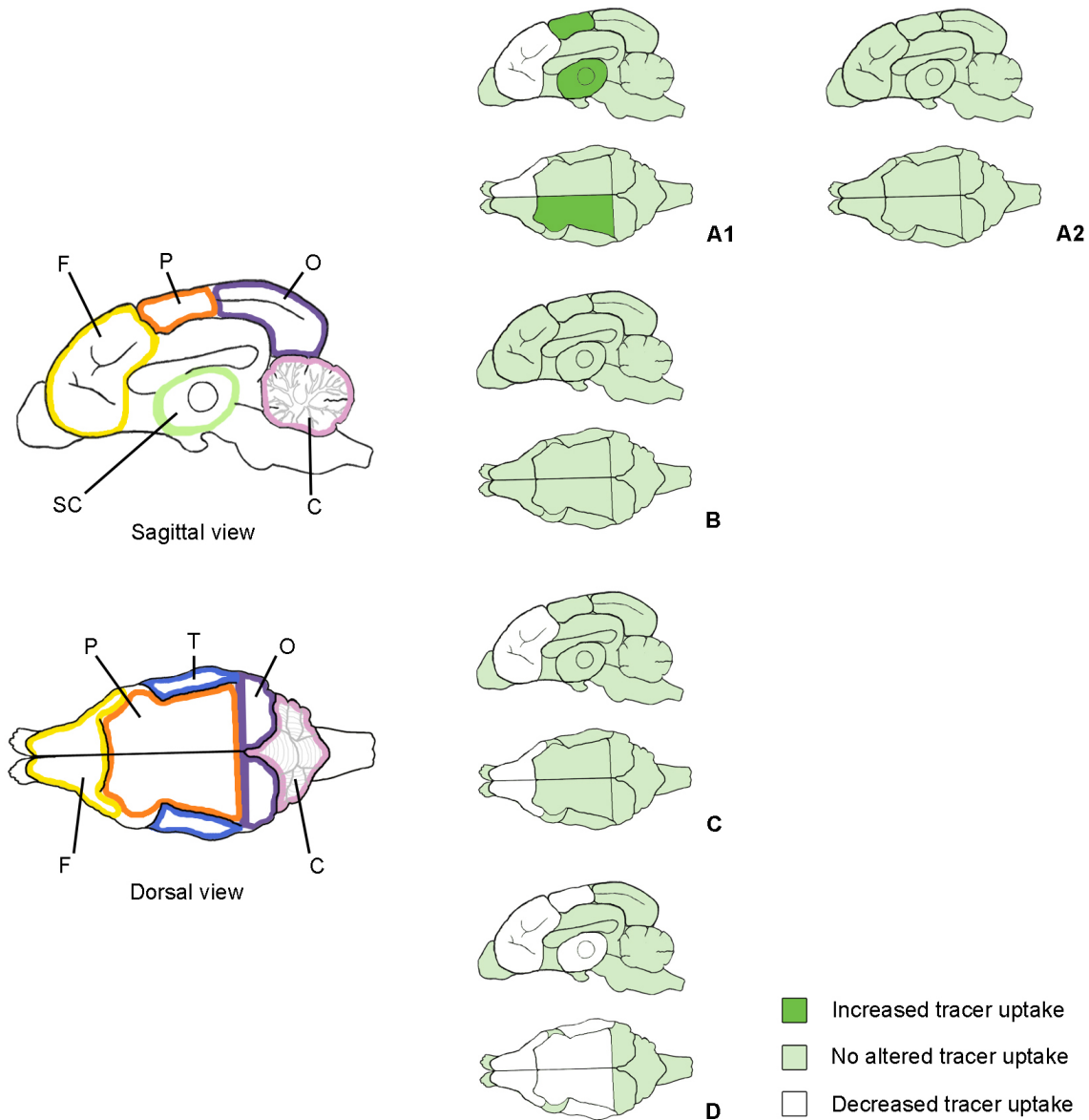


Figure 1. Schematic overview of the findings of the different research studies. On the left, an overview of the applied volumes of interest in two directions. On the right the results from the research studies. **A:** the effect of a single dose of morphine on regional cerebral blood flow (rCBF) at 30 minutes post-morphine (A1) and 120 minutes post-morphine (A2). **B:** the effect of prolonged morphine administration on rCBF. **C:** the effect of a single dose of morphine on the 5-HT_{2A} receptors. **D:** the effect of prolonged morphine administration on the 5-HT_{2A} receptors. F: frontal cortex, P: parietal cortex, O: occipital cortex, T: temporal cortex, SC: subcortical region, C: cerebellum.

A. Morphine and canine brain function

The first part of the present work investigated the effect of morphine on canine regional cerebral blood flow (rCBF) and, by analogy, brain function. The study regarding the effect of an acute administration of morphine (single intravenous dose of 0.5 mg/kg) on rCBF revealed two findings: alterations were found in the frontoparietal and subcortical region, and these changes in rCBF seemed to be time-dependent. Indeed, the decreased perfusion in the frontal cortex and the increased perfusion in the parietal cortex and subcortical region were only significant when comparing the data from 30 minutes after morphine administration to the baseline data. Prolonged administration of morphine (oral administration of 20 mg twice daily for 10 days), however, did not induce significant changes in rCBF, even though a trend of increased perfusion was found in the subcortical region.

The “acute” study revealed changes in cerebral perfusion in regions that are known to be part of pain processing, and additionally, that have been shown to be involved in opioid effects. The subcortical region contains important structures such as the brainstem and the thalamus. Both these brain structures play a key role in nociception and antinociception. The thalamus is a relay center for painful stimuli to be further processed by cortical regions and is therefore involved in the sensory-discriminative as well as the affective-motivational aspects of pain (Treede et al., 1999). The brainstem on the other hand, contains important structures for the descending control of pain. Both the thalamus and the brainstem were found to be activated or deactivated by pain and/or opioids (Wagner et al., 2007; Adler et al., 1997; Zelaya et al., 2012; Khalili-Mahani et al., 2011; Peyron et al., 2000; Casey et al., 1996; Casey et al., 2000). The changes observed in the left (and although not significant, right) parietal cortex are consistent with the activation of this region following a painful stimulus or opioid administration (Wagner et al., 2001; Peyron et al., 2000). The frontoparietal region is thought

to be involved in the attentional and associative components of pain processing (Ohara et al., 2005) and not surprisingly, brain activity in this region was altered in studies on pain and opioids (Wagner et al., 2001; Firestone et al., 1996; Khalili-Mahani et al., 2011; Adler et al., 1997). Accordingly, a study investigating the effects of remifentanyl on canine cerebral blood flow, found a decreased blood flow in the forebrain (Hoffman et al., 1993).

The seemingly time-dependent action of opioids on cerebral activity has been proposed before. In a study in dogs using remifentanyl, it was seen that cerebral blood flow and electroencephalogram values returned to baseline values after 30 minutes (Hoffman et al., 1993). Remifentanyl is a short-acting opioid, morphine on the other hand is a long-acting opioid, which could account for the delay in return to baseline values. Clinically, morphine is administered every 4 hours approximately, meaning that 2 hours after morphine administration, a clinical analgesic effect is still present, however, not visible in brain activity alterations. It is known that on top of its supraspinal action, morphine acts spinally as well as peripherally. Possibly the effects of morphine are more pronounced in the latter two areas as compared to the brain. Another possible explanation is the fact that once the MORs are activated by morphine, the downstream effects can linger on. A study investigating the temporal effects of remifentanyl in rats suggested that the temporal response to remifentanyl reflected underlying neurobiological processes (Liu et al., 2007). However, downstream effects (such as activation of other neurotransmitter systems) also require increased blood flow and oxygen supply, which does not match the observed lack of brain activity changes at 2 hours post-morphine, making this assumption less plausible. Future studies should look into this aspect further, adding more scanning time points.

In the “prolonged” study we failed to detect statistically significant changes in rCBF. Even though it is not advised to directly compare data obtained with ^{99m}Tc -ECD to ^{99m}Tc -HMPAO, it does correlate to the findings from the acute study, where no changes in rCBF were

detected 2h after morphine administration. However, the subcortical region did show a trend towards an increased perfusion after prolonged morphine administration. As mentioned, the subcortical region is important for pain processing and pain modulation. In this study we attempted to recreate an everyday situation using clinically relevant morphine doses and for a period of time that reflects a typical postoperative period necessitating analgesia. In research studies on the long-term effects of morphine and other opioids, the focus was mainly put on tolerance, addiction and dependence, which was not the aim of the study. Therefore, the results of our study are difficult to compare to those found in literature. Additionally, those addiction imaging studies are characterized by a great variation in study design, resulting, not surprisingly, in varying outcomes.

B. Morphine and the canine 5-HT_{2A} receptor system

The second part of the present work investigated the possible effect of morphine on the canine cerebral 5-HT_{2A} receptor system. First, a single acute morphine dose was administered which generated a decreased 5-HT_{2A} receptor binding in the frontal cortex. In the second study, a prolonged exposure to morphine also decreased 5-HT_{2A} receptor binding in the frontal cortex, and, additionally, in the temporal and parietal cortices and the subcortical region. It is important to note that the observed changes were not merely due to alterations in tracer delivery due to morphine-induced blood flow changes since the rCBF studies provide evidence that morphine does not influence regional brain perfusion at these time points.

In the “acute” study, we observed a decreased 5-HT_{2A} receptor binding in the frontal cortex. 5-HT_{2A} receptors are widely distributed throughout the cortex, with particularly high densities in the frontal area (Pompeiano et al., 1994; Burnet et al., 1995; López-Giménez et al., 1998; Hall et al., 2000; Amargós-Bosch et al., 2004). The MOR as well is highly

concentrated in the frontal cortex and both receptors have been shown to be co-expressed in frontocortical layer VIb (López-Giménez et al., 2008). The exact reason for the decreased binding in the frontal cortex cannot be determined by the present results, however several theories can be proposed. First, it is well established that morphine analgesia relies in part on the serotonergic system. Morphine increases 5-HT release in several brain regions and this increase in synaptic 5-HT might result in two distinct receptor mechanisms: desensitization and down regulation. Desensitization is a rapid process that follows activation of a receptor by an agonist, this can be homologous (by the receptor ligand, in this case 5-HT) or heterologous (by a ligand that binds to another GPCR, in this case morphine) (Gray and Roth, 2001; Van Oekelen et al., 2003). 5-HT_{2A} receptor desensitization has been observed in vitro as soon as 15 minutes after 5-HT exposure (Van Oekelen et al., 2001). Heterologous desensitization has been described for the MOR and 5-HT_{2A} receptors, namely, MOR receptors were co-activated when 5-HT_{2A} receptors were activated by 5-HT (López-Giménez et al., 2008). In the present study, this might be interpreted the other way around with morphine being the co-activator/heterologous desensitizer for the 5-HT_{2A} receptor. However, the radioligand used in the present studies is a neutral antagonist that binds to both the active receptor and the inactive (or desensitized) receptor. The radioligand can therefore not distinguish between both receptor states and thus 5-HT_{2A} receptor desensitization is less likely to be a plausible explanation for our findings. Another possibility is the down regulation of the 5-HT_{2A} receptor following 5-HT release. This is a slow process that leads to a decreased number of receptors on the plasma membrane, typically following repeated exposure to an agonist. However, in the same study of Van Oekelen and colleagues, it was demonstrated that cell treatment with 5-HT for 15 minutes and 48 hours did not result in decreased 5-HT_{2A} receptor numbers (Van Oekelen et al., 2001). Direct binding of morphine to 5-HT_{2A} receptors could also be possible since similar interactions between other MOR

agonists and 5-HT receptors have been described (Wittmann et al., 2006; Tao et al., 2003). However, the affinity of morphine for the 5-HT_{2A} receptor is rather low (Leysen et al., 1982). Conformational changes in the 5-HT_{2A} receptor is another possibility, causing a decreased affinity for the radioligand. Receptor heterodimerization is less likely since the 5-HT_{2A} receptor and the MOR do not share a common cellular distribution (López-Giménez et al., 2008).

In the “prolonged” study decreased 5-HT_{2A} receptor binding was seen in almost the entire canine brain. This suggests that prolonged morphine has a greater impact on the serotonergic system. Additionally, prolonged or repeated exposure of GPCRs to their agonists leads to receptor down regulation due to increased receptor internalization and degradation. This has been observed for the 5-HT_{2A} receptor following prolonged agonist exposure in several *in vivo* and *in vitro* studies (review: Van Oekelen et al., 2003). In the treatment of 5-HT-related brain diseases such as depression, a prolonged period of treatment is usually required in order to obtain a sufficiently high 5-HT tone. This is because the increased 5-HT tone in itself is not the key to treatment, but rather the downstream neuronal adaptations that result from these increased synaptic 5-HT levels, such as decreased 5-HT_{2A} receptor numbers (Manji et al., 2000; Stahl, 2008). In depressed patients, adaptive upregulation of postsynaptic receptors, such as the 5-HT_{2A} receptor, has been observed (Shelton et al., 2009) and a decreased number of 5-HT_{2A} receptors is seen after antidepressant treatment both in humans and dogs (Gray and Roth, 2001; Peremans et al., 2005).

As mentioned, MOR agonists are known to increase brain 5-HT levels, thus mimicking the effect of common antidepressant drugs like selective serotonin reuptake inhibitors (SSRIs). This raised the question whether opioids could be potential antidepressants in addition to traditional treatment that does not always provide full recovery. Indeed,

biochemical and behavioral studies suggest the involvement of the opioid system in the mechanism of action of certain antidepressants (Fichna et al., 2007b). Additionally, opioids have been found to have significant antidepressant-like properties in both rodent models of depression and in humans suffering from treatment-refractory depression (Bodkin et al., 1995; Tejedor-Real et al., 1995; Stoll and Reuter, 1999). However, the exact potential of opioids as antidepressant agents remains a matter of discussion. In a recent PET imaging study designed to map connections between neurotransmitter systems, regionally specific intercorrelations were observed between the opioid and serotonin system in regions that are known to play a role in several neuropsychiatric diseases (Tuominen et al., 2013). Our results contribute to this knowledge, given that 5-HT_{2A} receptor binding was decreased after morphine treatment (both “acute” and “prolonged”), which is one of the objectives of the treatment of neuropsychiatric disease with a 5-HT_{2A} receptor component (e.g.: effect of antidepressants on 5-HT_{2A}: Meyer et al., 2001; antidepressants augmented by atypical antipsychotics: Philip et al., 2008).

With regards to the role of the serotonergic system – and the 5-HT_{2A} receptor in particular – in opioid effects and side effects, our results support the existence of a link between the two systems (opioid and serotonergic) and provide ground for further investigation into the specific role of the 5-HT_{2A} receptor in this interaction. To enhance the therapeutic effects of opioids, one possible option is to target other neurotransmitter systems that are involved in opioid analgesia, such as the serotonergic system. Adding antidepressants that augment cerebral 5-HT levels is one option that has been proven successful (Nayebi et al., 2009; Banks et al., 2010); specifically targeting certain 5-HT receptors is another. In the light of the present results and given that 5-HT_{2A} receptor antagonists have been seen to have analgesic properties (Nishiyama, 2005), the 5-HT_{2A} receptor might be one of those targets. As for the role of the 5-HT_{2A} receptor in long-term opioid side effects, results are

not that straightforward. Sandrini and colleagues found that although morphine and paracetamol decreased 5-HT₂ receptor binding, only morphine induced tolerance in vivo suggesting that the 5-HT₂ receptors were not involved in the development of tolerance (Sandrini et al., 2007). On the other hand, López-Giménez and colleagues found that 5-HT_{2A} receptor co-activation in vitro reduces morphine tolerance by co-activation of intracellular pathways (López-Giménez et al., 2008). In addition, since morphine causes abnormal/diminished internalization of the MOR (see introduction), promoting MOR internalization could be an important tool to counter the development of tolerance, as was observed by López-Giménez and colleagues (López-Giménez et al., 2008). In this light, our results give further hints towards that end, as there appears to be a visible link between both systems. With regards to addiction, 5-HT_{2A} antagonists were found to prevent the hyperreactivity of the serotonergic and noradrenergic system associated with repeated administration of drugs of abuse such as morphine (Lanteri et al., 2008).

C. Methodological considerations

Two different brain perfusion tracers were used for the studies on the effects of morphine on canine brain function, namely ^{99m}Tc-ECD for the acute study and ^{99m}Tc-HMPAO for the prolonged study. As mentioned in the introduction of this thesis, both of these perfusion tracers are commonly used in human investigations, however, until recently, ^{99m}Tc-ECD was mainly used in canine studies. Due to the suspended production of ^{99m}Tc-ECD, we investigated the possibility of using ^{99m}Tc-HMPAO as an alternative for ^{99m}Tc-ECD. Since studies in human research found differences in cerebral distribution patterns between both tracers, we investigated this question in dogs. Indeed, comparison of those perfusion tracers in dogs revealed regional differences. The use of ^{99m}Tc-ECD resulted in a higher perfusion index in the subcortical region compared to ^{99m}Tc-HMPAO. On the other hand, the perfusion

index was significantly lower in the cerebellum in the ^{99m}Tc -ECD data compared to the ^{99m}Tc -HMPAO data. These data support the use of both tracers for cerebral perfusion evaluation in dogs, however, they are not interchangeable and direct comparison is not always advised. Additionally, since the cerebellum is commonly used as a reference region for semiquantification purposes, the difference in uptake between both tracers is of great importance and semiquantification using the entire brain as a reference region is more reliable.

There are six concerns that need to be addressed with regards to the present work: the pharmacokinetics of oral morphine in dogs, the so-called prolonged 10-day morphine treatment, the absence of pain in the dogs, the need for anesthesia during scanning, the gender, and the clinical relevance of the present findings.

In the studies investigating the effect of prolonged administration of morphine on the canine brain, this prolonged treatment consisted of an oral sustained release formulation of morphine (20 mg morphine sulphate, MS Contin[®], Mundipharma, Mechelen, Belgium) administered orally twice daily for 10 days, which is comparable to a dosage of 2.37 ± 0.25 mg/kg (perfusion study) and 2.86 ± 0.16 mg/kg (5-HT_{2A} receptor study) twice daily. The oral bioavailability of morphine in dogs is poor due to the first pass elimination by liver and intestinal mucosa with the liver being the major site of morphine metabolization in dogs (Merrell et al., 1990). Therefore, morphine concentrations were measured in plasma (both studies) and in cerebrospinal fluid (CSF) (only in the perfusion study) to ensure adequate dosage. In the brain perfusion study, mean plasma concentrations were 47.60 ± 9.74 ng/mL and mean CSF concentrations were 9.53 ± 3.12 ng/mL. In the 5-HT_{2A} receptor study, mean morphine plasma concentrations were 29.8 ± 7.8 ng/mL. These concentrations are comparable to the concentrations described in dogs that are clinically relevant – i.e. that produce

analgesia – after similar treatment (Hug, 1981; KuKanich et al, 2005; Dohoo, 1997). As was described in the study by Dohoo, we also found a high intersubject variability (Dohoo, 1997).

For the duration of the prolonged morphine administrations, we attempted to recreate a situation that was close to everyday clinical settings, where postoperative pain management requiring opioids rarely exceeds 10 days. It is important to underline that we did not attempt to induce tolerance in those dogs, as higher doses or a longer period would have been needed (Yoshimura et al., 1993). Additionally, tolerance would have needed to be assessed by other methods, such as measuring paw withdrawal latencies after a painful stimulus, in order to be certain that tolerance to morphine was present. This leads us to another important reflection, the fact that the dogs in the present studies were non-painful dogs. As mentioned in the introduction chapter, chronic pain can cause functional as well as structural alterations. Taking this into consideration, painful dogs could respond differently to morphine and show different imaging results.

As mentioned in the introduction, imaging studies in animals have one important disadvantage, the need to immobilize them in order to be able to perform the image acquisition. This is mainly achieved by anesthetizing the animals. This could possibly have an influence on the neurotransmitter system under observation and additionally on cerebral blood flow, with potential influence on the distribution of the radioactive tracer. These influences always need to be taken into account when interpreting the obtained data. Studies from our research group have examined the impact of several anesthetics on brain function and the 5-HT_{2A} receptor system. To put in a simple way, the following overall conclusions can be drawn from these studies. For the brain function studies: since the tracer provides a fixed image because it is trapped in the brain cells within 2 minutes, all drugs administered after the plateau is reached have no influence on the data. For the 5-HT_{2A} receptor studies:

the type of anesthetics have to be considered in function of their possible interaction with the 5-HT system, and second, as long as we adhere strictly to the same anesthetic protocol, differences observed between two study groups should be due to the interfering factor (in the present case: the administration of morphine).

In this line, it is also important to work with homogeneous groups of subjects. Sex differences are known to exist in nociception and in the response to opioids (Kest et al., 2000; Hurley et al., 2008). We opted for neutered female dogs since this would overcome the cyclic hormonal influences of intact female dogs. However, morphine appears to be less potent in females (Lloyd et al., 2008), thus it would be interesting for future studies to compare the present results to new studies in male dogs as the finding might be more pronounced. Ageing has been shown to have an influence on cerebral 5-HT_{2A} receptors; therefore, it is advised to use groups with similar ages (Baeken et al., 1998; Peremans et al., 2002).

Last but certainly not least, it is important to be able to determine the clinical relevance of our findings. Statistically significant does not always mean clinically relevant and vice versa. This is difficult to assess correctly, often requiring an extra dimension to the obtained imaging results, such as behavioral data. For the 5-HT_{2A} receptors indeed, a study on canine impulsive aggression revealed improvement of the aggressive behavior after treatment, together with decreased 5-HT_{2A} receptor binding (Peremans et al., 2005). The magnitude of the differences in binding was comparable to those in the present studies, providing solid ground for the clinical relevance of our results.

D. Conclusions and future studies

We investigated the influence of morphine on the canine brain, more specifically, on cerebral perfusion and 5-HT_{2A} receptors. As expected, morphine altered the brain perfusion, and thus, brain function. Unexpectedly, these changes appear to be time-dependent. In

addition, morphine alters 5-HT_{2A} receptor availability. This was more pronounced in the prolonged study thus emphasizing the 5-HT_{2A}-opioid interaction, which could be of importance for mood disorder treatment on the one hand and opioid long-term side effects on the other hand.

The present work underlines the value of brain imaging in drug research and strengthens the use of SPECT in dogs. An important aspect of drug research is inevitably the need for animal models and providing in vivo models that give a maximum of information with the least amount of discomfort, which is achievable with brain imaging studies. To progress in drug research it is important to first gain as much knowledge as possible on the presently available drugs so that a complete image is created, which can be used as a soundboard for new discoveries. To gain insights into the modes of action of a protagonist opioid such as morphine is therefore of great value and future studies on the topic should be considered.

Future imaging studies could include comparing the rCBF pattern of morphine to patterns generated by other opioids. These studies could also include shorter time intervals between scans to further investigate the temporal differences seen with morphine. Other dosing protocols could also provide valuable information. In this light, inducing morphine tolerance could be an interesting research path to elaborate, which can be combined with a behavioral component, such as paw withdrawal latency tests, to assure tolerance is present. On the other hand, seeing as other neurotransmitter systems are closely involved in the working mechanism of opioids, it could be interesting to look at their reaction to morphine. For instance, dopamine is known to be an important factor in opioid side effects and the influence of prolonged morphine administration on the dopaminergic system could be investigated.

Next to studies in healthy dogs, dogs suffering from chronic pain could also provide important information. Chronic pain does not only have a debilitating effect due to the

ongoing pain, it also has an impact on mental health. Depression is often seen in patients with chronic pain. Therefore, rCBF and 5-HT_{2A} receptor availability could be assessed in these patients as well as the influence of opioids and other drugs. Ketamine, for instance, is used as an analgesic – interestingly, as an adjunct to opioid therapy as well – and has been shown to possess antidepressant properties (review: Murrough, 2012). In a previous study from our research group, ketamine was found to decrease 5-HT_{2A} receptor availability in the frontal and temporal cortices in cats (Waelbers et al., 2013) and future studies on this topic should be developed.

SUMMARY



Regional cerebral blood flow (rCBF) is known to be a measure for regional brain activity. Therefore, it can be used to determine how a drug works by examining the rCBF pattern it produces. On top of this, different drugs can be compared by comparing their rCBF patterns. In the case of analgesics, it is interesting to compare the possibly different loci of activity depending on the analgesic drug. To evaluate rCBF in dogs, previous studies have used ^{99m}Tc -ECD single photon emission computed tomography (SPECT). However, in human medicine, ^{99m}Tc -HMPAO has also been frequently used in brain imaging studies. Therefore, the first aim of the present doctoral thesis was to compare both ^{99m}Tc -ECD and ^{99m}Tc -HMPAO for rCBF evaluation in dogs.

1. The comparison of rCBF evaluation with ^{99m}Tc -ECD and ^{99m}Tc -HMPAO in dogs

The aim of the first study was to examine whether regional distribution differences between both tracers occur in dogs as is reported in humans. Eight beagles underwent two SPECT examinations first with ^{99m}Tc -ECD and followed by ^{99m}Tc -HMPAO. Changes in rCBF between both tracers were found in the subcortical ($P = 0.001$, $\text{ECD} > \text{HMPAO}$) and to a lesser extent in the cerebellar region ($P = 0.03$, $\text{ECD} < \text{HMPAO}$). This study shows that both tracers can be used for the visualization of the canine cerebral blood flow. However, due to differences in regional distribution, a selection of either ^{99m}Tc -ECD or ^{99m}Tc -HMPAO should be made and, most importantly, direct comparison between both tracers is not recommended.

A prototypical analgesic drug frequently used in both veterinary and human medicine is the mu opioid receptor (MOR) full agonist, morphine. It is widely used in cases of acute pain such as perioperative pain management. The aim of the second section of this doctoral thesis was therefore to examine the effect of morphine on the canine rCBF to gain insights on how this opioid works in dogs.

2. The influence of a single dose of morphine on canine rCBF

The aim of the second study was to determine whether a single intravenous bolus of morphine (0.5 mg/kg) alters rCBF. This experimental randomized cross-over study was conducted in 8 beagle dogs. Three SPECT scans were performed using ^{99m}Tc -ECD: Baseline, 30 minutes after morphine and 120 minutes after morphine. We found a significant increase in rCBF in the subcortical region and the parietal region and a decrease in the frontal region in the scan 30 minutes after morphine compared to the baseline values. The subcortical region includes structures important for pain modulation (inhibition) and therefore it is not surprising that an analgesic would increase brain activity in this region. However, we could not find differences 120 minutes after morphine. This could possibly imply a time-dependent mode of action of morphine, which would be interesting to investigate more thoroughly in future studies.

3. The influence of prolonged morphine administration on canine rCBF

The aim of the third study was to evaluate the effect of a 10-day morphine treatment on cerebral perfusion. We conducted a randomized cross-over study in 8 beagle dogs. Two SPECT scans were performed to evaluate the rCBF using ^{99m}Tc -HMPAO: Baseline and after 10 days of morphine treatment (20 mg twice daily, oral sustained release morphine sulphate). Morphine plasma concentrations were also determined on the last day of treatment to evaluate whether the administered morphine dose was sufficient. Comparison of the Baseline and the morphine perfusion failed to reveal significant differences. Plasma concentrations were comparable to those in literature and are considered to be clinically relevant.

An interaction between the opioid and serotonergic system has long been described. Indeed, opioids can increase serotonin (5-HT) levels in the spinal cord (descending pain inhibition) but also in the brain. This interaction at a supraspinal level is important not only

for the analgesic properties of opioids, but it can also account for 1) the possible antidepressant effect of opioids (increasing serotonin levels is a key component of antidepressant therapy), and 2) the development of long term side effects of opioids (downstream alterations of neurotransmitter systems is an important causal effect). More specifically, the 5-HT_{2A} receptor has been reported to play a role in analgesia, depression and in the development of opioid side effects. Therefore, the aim of the third section of this thesis was to investigate the influence of morphine in the canine cerebral 5-HT_{2A} receptors measured with ¹²³I-R91150 SPECT.

4. The influence of a single dose of morphine on canine cerebral 5-HT_{2A} receptors

The aim of the fourth study was to assess the influence of a single intravenous bolus of morphine (0.5 mg/kg) on cerebral 5-HT_{2A} receptor binding in dogs using single SPECT with the 5-HT_{2A} radioligand ¹²³I-5I-R91150. The 5-HT_{2A} receptor binding was estimated with and without morphine pre-treatment in 8 beagle dogs. A significantly decreased 5-HT_{2A} receptor binding was found after morphine in the right ($P = 0.012$) and left ($P = 0.040$) frontal cortices. Our findings support the existence of an interaction between morphine and 5-HT_{2A} receptor binding in the canine frontal cortex. In the absence of pain and mood disorders, morphine decreased the 5-HT_{2A} receptor availability. Whether this is caused by decreased receptor density due to direct internalization or the result of indirect actions, such as increased endogenous serotonin release, remains to be elucidated.

5. The influence of prolonged morphine administration on canine cerebral 5-HT_{2A} receptors

The fifth study examined the effect of prolonged opioid treatment on cerebral 5-HT_{2A} receptors. Cerebral 5-HT_{2A} receptor availability was estimated in 7 beagle dogs pre and post 10-day morphine treatment (20 mg twice daily oral sustained release morphine) with ¹²³I-R-91150, a 5-HT_{2A} selective radioligand, and SPECT. Morphine treatment significantly ($P \leq$

0.05) lowered 5-HT_{2A} receptor binding in the right and left frontal cortex, the right and left temporal cortex, the right and left parietal cortex, and the subcortical region. Prolonged exposure to morphine decreased the 5-HT_{2A} receptor availability in a large part of the canine cortex. This finding gives further evidence for the existence of an interaction between the opioid and 5-HT_{2A} receptor system and encourages further research on the possible beneficial effects of opioids in the treatment of mood disorders.

SAMENVATTING



Het bepalen van regionale hersendoorbloeding is een maat voor hersenactiviteit en kan dus gebruikt worden om te achterhalen hoe bepaalde geneesmiddelen werken door te kijken naar het regionale doorbloedingspatroon dat een bepaald geneesmiddel creëert. Het biedt daarenboven eveneens de mogelijkheid om verschillende geneesmiddelen met elkaar te vergelijken door het vergelijken van de geproduceerde doorbloedingspatronen. Dit is in het geval van pijnstillers (analgetica) bijzonder interessant. Om de regionale hersendoorbloeding bij honden te evalueren hebben voorgaande studies gebruik gemaakt van functionele beeldvorming (SPECT) met behulp van de radioactieve tracer ^{99m}Tc -ECD. In de humane geneeskunde wordt naast ^{99m}Tc -ECD, het andere radioligand ^{99m}Tc -HMPAO ook frequent aangewend voor het bepalen van de regionale hersendoorbloeding en werden deze twee radioliganden reeds vaak met elkaar vergeleken. Bij de hond werd dit tot hiertoe nog niet gedaan en daarom was de **eerste doelstelling van deze doctoraatsthesis** het vergelijken van ^{99m}Tc -ECD en ^{99m}Tc -HMPAO voor de evaluatie van regionale hersendoorbloeding bij de hond.

1. Vergelijking van de regionale hersendoorbloeding met ^{99m}Tc -ECD en ^{99m}Tc -HMPAO bij de hond

De doelstelling van de eerste studie was onderzoeken of de regionale distributiever verschillen tussen de twee radioliganden die gezien worden bij de mens, ook bij de hond voorkwamen. Acht beagles werden tweemaal onderzocht d.m.v. SPECT, eerst met ^{99m}Tc -ECD, gevolgd door ^{99m}Tc -HMPAO. Er werden verschillen vastgesteld tussen beide radioliganden in de subcorticale regio ($P = 0.001$, $\text{ECD} > \text{HMPAO}$) en in mindere mate in het cerebellum ($P = 0.03$, $\text{ECD} < \text{HMPAO}$). Deze studie toont aan dat beide radioliganden gebruikt kunnen worden voor de bepaling van regionale hersendoorbloeding. Het is echter aangewezen om een keuze te maken tussen beide radioliganden gezien de regionale verschillen en een rechtstreekse vergelijking van data tussen de twee radioliganden is niet aan te raden.

De volle mu opiaat receptor agonist morfïne is een voorbeeldpijnstiller zowel in de humane geneeskunde als de diergeneeskunde. Het wordt frequent aangewend in gevallen van acute pijn, zoals bijvoorbeeld in het geval van perioperatieve pijnbestrijding. De **tweede doelstelling van deze doctoraatsthesis** was daarom het onderzoeken van de invloed van morfïne op de regionale hersendoorbloeding om zo meer inzicht te krijgen in het werkingsmechanisme van dit opiaat bij de hond.

2. De invloed van een éénmalige morfïne toediening op de regionale hersendoorbloeding bij de hond

De doelstelling van de tweede studie was het onderzoeken van het effect van een éénmalige intraveneuze toediening van morfïne (0.5 mg/kg) op de regionale hersendoorbloeding. Het onderzoek werd uitgevoerd bij 8 beagles volgens een gerandomiseerd cross-over design. Er werden telkens 3 SPECT scans gedaan m.b.v. ^{99m}Tc -ECD: baseline, 30 minuten na morfïne en 120 minuten na morfïne. Er werd een significante stijging van de hersendoorbloeding gevonden in de subcorticale regio en de pariëtale regio en een daling in de frontale regio in de scans 30 minuten na morfïne vergeleken met de baseline scans. De subcorticale regio bevat structuren die belangrijk zijn voor pijnmodulatie (o.a. pijn inhibitie) en het is daarom niet verrassend dat een pijnstillers in deze regio veranderingen veroorzaakt. Dit verschil in doorbloeding was echter niet terug te vinden in de scans 120 minuten na morfïne wat mogelijks zou kunnen wijzen op een tijdsafhankelijk effect van morfïne. Dit eventuele tijdsafhankelijk effect zou een interessante verdere onderzoekspiste kunnen zijn voor latere studies.

3. De invloed van een langdurige toediening van morfïne op de regionale hersendoorbloeding bij de hond

Het doel van de derde studie was het onderzoeken van het mogelijke effect van een 10-daagse morfine toediening (20 mg/kg BID orale vertraagde vrijstelling morfine) op de regionale hersendoorbloeding. Deze cross-over studie werd uitgevoerd bij 8 beagles op een gerandomiseerde wijze. Er werden telkens 2 SPECT scans uitgevoerd m.b.v. ^{99m}Tc -HMPAO: baseline en na 10 dagen morfine. Op de laatste dag van de morfinebehandeling werden eveneens plasmaconcentraties van morfine bepaald om na te gaan of de gebruikte morfine dosering voldoende was. De vergelijking van de hersendoorbloeding met en zonder 10 dagen morfine toonde geen significante verschillen aan. De plasmaconcentraties van morfine waren vergelijkbaar met deze gevonden in de literatuur en zijn klinisch relevant.

Het is reeds lang gekend dat er een interactie bestaat tussen het opiaat en het serotonerge systeem ter hoogte van het ruggenmerg (descenderende pijninhiberende banen), maar ook ter hoogte van de hersenen. Deze zogenaamde supraspinale interactie is niet alleen belangrijk voor de analgetische eigenschappen van opiaten, maar kan eveneens een rol spelen 1) in de mogelijke antidepressieve werking van opiaten (verhoogde 5-HT vrijstelling in de hersenen is een hoofdonderdeel van de behandeling van depressie) en 2) in het ontstaan van nevenwerkingen door het chronische gebruik van opiaten (downstream-veranderingen in bepaalde neurotransmittersystemen wordt gezien als een belangrijk onderdeel van de ontwikkeling van deze nevenwerkingen). In het bijzonder werd gezien dat de 5-HT_{2A} receptor een mogelijke rol speelt in alle drie de voornoemde onderdelen: analgesie, depressie en opiaatnevenwerkingen. Daarom was de doelstelling van het **derde onderdeel van deze doctoraatsthesis** het onderzoek naar de invloed van morfine op het 5-HT_{2A} receptorsysteem in de hersenen van de hond m.b.v. SPECT.

4. De invloed van een eenmalige toediening van morfine op 5-HT_{2A} receptoren in de hersenen bij de hond

De doelstelling van de vierde studie was het onderzoeken van de mogelijke veranderingen in 5-HT2A receptorbinding door een eenmalige toediening van morfine (0.5 mg/kg intraveneus). De 5-HT2A receptorbinding werd gemeten d.m.v. SPECT en het 5-HT2A receptorspecifieke radioligand ^{123}I -R-91150. Bij 8 beagles werden telkens 2 SPECT scans uitgevoerd: met en zonder morfine. In de linker en rechter frontale cortex werd een significante daling van de 5-HT2A receptorbinding gevonden in de scans na morfine (rechts $P = 0.012$, links $P = 0.040$). Deze bevindingen ondersteunen het bestaan van een opiaat-serotonine-interactie in afwezigheid van pijn en/of gemoedsproblemen. De exacte aard van deze interactie dient verder uitgediept te worden aangezien met deze studie geen onderscheid gemaakt kan worden tussen een directe of indirecte interactie tussen morfine en de 5-HT2A receptoren.

5. De invloed van een langdurige toediening van morfine op de 5-HT2A receptoren in de hersenen van de hond

De doelstelling van de vijfde studie was het onderzoek naar het effect van een 10-daagse morfine toediening (20 mg/kg BID orale vertraagde vrijstelling morfine) op het 5-HT2A receptorsysteem. De 5-HT2A receptorbinding voor en na 10 dagen morfine werd geëvalueerd bij 7 beagles d.m.v. SPECT en het 5-HT2A receptorspecifieke radioligand ^{123}I -R-91150. De 10-daagse morfine toediening verlaagde de 5-HT2A receptorbinding significant ($P \leq 0.05$) in de linker en rechter frontale, linker en rechter temporale, linker en rechter pariëtale cortex en de subcorticale regio. Het 5-HT2A receptorsysteem werd in een groot deel van de hersenen beïnvloed door langdurige morfine toediening wat verder bewijs geeft voor een link tussen het opiaat en serotonerge systeem. Dit moedigt verder onderzoek aan naar andere mogelijke interacties tussen beide systemen voor een zoektocht enerzijds naar een betere pijnbestrijding en anderzijds naar een betere behandeling voor gemoedsstoornissen zoals depressie.

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CURRICULUM VITAE



Antita Adriaens werd geboren op 24 februari 1984 in Edegem. Ze behaalde haar diploma secundair onderwijs aan het Instituut Heilige Familie in Berchem en startte hierna in 2002 met de studies Diergeneeskunde, eerst aan de UA en vervolgens aan de UGent. In 2008 behaalde ze het diploma van dierenarts met grote onderscheiding.

Meteen na haar studies begon ze aan dit doctoraatswerk op de dienst anesthesie van de vakgroep Geneeskunde en Klinische Biologie van de Kleine Huisdieren in samenwerking met de dienst nucleaire diergeneeskunde van de vakgroep Medische Beeldvorming van de Huisdieren en Orthopedie van de Kleine Huisdieren. In het kader van dit onderzoek volgde ze eveneens de doctoraatsopleiding van de Doctoral Schools waarvoor ze verschillende cursussen volgde en ook het diploma van proefleider (FELASA categorie C) behaalde.

Antita Adriaens is auteur en mede-auteur van verschillende wetenschappelijke publicaties. Ze nam deel aan verschillende nationale en internationale congressen als spreker en als mede-auteur.

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Dankwoord



08:27 woensdag 23 oktober, de dakmannen zijn eindelijk de stormpan op het dak aan het plaatsen. Geen idee wat het eigenlijk is of doet, want we hebben al 2 jaar kunnen overleven zonder. Onder het motto “beter laat dan nooit” zal ik dan ook maar eens aan dit laatste deeltje beginnen.

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je nog verbouw-info nodig hebt en misschien komen we mekaar eindelijk eens tegen op een concertje!

Nu over van de rotsen en de branding naar de rustige oase: de bureau! Ondertussen is de bezetting alweer veranderd, maar altijd even fijne mensen! Ik denk dat iedereen wel weet dat we een leutige bureau hebben, ook zonder decibelmeter in de hand. Valentine, de mama (letterlijk en figuurlijk) van de bureau. Onze voorliefde voor het uitwisselen van nieuwtjes was misschien niet altijd even productief, maar goed, dat maakte het bureauleven wel interessanter! Je bent er ook bijna, komt allemaal goed! Steven, uiteraard was jij de fijnste bureaucollega, al was het maar wegens jouw onberispelijke smaak voor muziek! Nogmaals succes met je examen! An, wat ik van jou geleerd heb, naast het koken van artisjokken, te laat komen in de cinema en het verloren lopen tijdens wandelingen, is dat geen enkel idee te gek is! Ine, ook altijd paraat voor een babbel en een wandel! Ik herinner me Sali the early years nog, met als hoogtepunt de gemolde bench! Ongelooflijk vond ik dat! Of nog, de grote sprong! Maar wat een schatje is het! Je hebt me ook altijd hoop gegeven met de 2-jaar-regel... ik heb daar zo mijn theorie over ontwikkeld ondertussen, maar dat is voor een andere keer! We moeten dringend nog eens bijkletsen! Daniela, living the American Dream! Zoals beloofd, een boekje opgestuurd! Een aaitje aan al jouw beestjes daar!

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Matthias y Cata, voy a probar de decirlo en español! Estoy muy contenta que he conocido a vosotros y que, cuando nos vemos, siempre está una fiesta! Oh boy, I really should take some lessons again, because this really is too basic! Anyway, I am so happy to have you amongst my friends and I really look forward to going to Colombia with you one day! In the mean time, I hope we can enjoy many Lucy changs and Oud Kloosters together!

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uitkijken naar New York! Nicky, de kleine broer (hahaha, sorry ik kon het niet laten)! Veel plezier daar in Cuba! Breng wat rum mee en we vieren nog wel eens als jullie terug zijn!

Et puis, la famille! Et elle est grande notre famille! (je m'excuse déjà d'avance pour les fautes d'orthographe. Enfin, j'espère quand-même qu'il y en aura pas trop). Alors, ou commencer? Grand-mère, je commence avec toi, vu que tu es au top de la famille! Ça m'a toujours fait plaisir de venir étudier chez toi, les bons petits repas (on mange avec les yeux comme tu dis toujours) et les pauses Roland-Garros (vive Nadal!)? On en a eu des discussions, toujours très intéressantes. Bien que je dois avouer que ta confiance en l'homeopathie reste difficile à comprendre. Je t'embrasse très fort!

Alors maintenant, 'les petits'? Vous n'êtes plus tellement petits que ça et ça me fait encore toujours drôle de penser qu'on vieillit tous ensemble. En tout cas je peux dire que je suis vraiment fière d'être votre grande sœur et je vous aime tous énormément! Timo, mon petit frère, arrête de faire le Goron! En avant, marche! (et coupe tes cheveux! ;-)) Magali-tje, j'ai encore souvent pensé à toi ces derniers temps à propos de ce que tu disais sur 'se rencontrer soi-même'. On s'accroche pour la belle aventure: 'tu vis ta vie et tu t'en sors' (comme disait Tom Barman). Prochaine fois que Buraka passe: tu viens avec! ("avec est un petit bonhomme qui se promène jamais tout seul", mais je m'en fous :-)) Nuwan, Nunneke, tu vas finir par trouvé le truc génial, nec plus ultra qui tue a mort! Avec ton sense du business! :-)) Matteo, trois mots pour toi: YES YOU CAN! Et puis enfin, Manon! Aaah! L'enfant terrible! (je rigole) C'est bête que tu ne sois pas là aujourd'hui! Tu ne peux pas savoir à quel point je suis contente que tu m'as aidé ces derniers mois! Sans toi, je ne sais pas à quoi aurait ressemblé mon petit livre... (je n'ose même pas y penser) Merci merci merci!!!

Comme ça on arrive à la source! Mamy! Je ne sais pas très bien quoi dire à vrai dire... J'ai pas envie de tomber dans les clichés à deux balles, tu comprends. Ou peut-être qu'il faut y aller à fond! En tout cas, je ne crois pas qu'il y ait une meilleure maman sur la terre, dans la

galaxie, dans l'univers! 🎵 Maman, c'est toi, la plus belle du monde! 🎵 Oh non, c'est vraiment over-the-top! Je crois que le plus important c'est: Merci de nous avoir fait! Tout les six! Et, tu ne t'en rends peut-être pas compte, de nous avoir donné une enfance, pas comme les autres, mais meilleure! Na-na-nère!

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